OFFICE OF PROCUREMENT
2-STEP REQUEST FOR BID (RFB): INSTRUCTIONAL MATERIAL DESIGN & BINDING SERVICES
RFB NUMBER: 919-007
RFB CLOSING DATE: May 22, 2019 @ 3:00PM

MONTGOMERY COLLEGE

ADDENDUM #1
Issued: May 16, 2019

ADDENDUM FOR THE PURPOSE OF:

- Delete the following statement from section 2.2 (Scope of Services):

  "Interactions with guest presenters may require 24-48hour turnaround with slides and student materials."

- Change section 2.3 (Deliverables) to the following:

  "Service deliverable includes two fully loaded thumb drives and the designated quantity of student workbooks delivered at a mutually agreed up shipping and delivery date. Typical workflow: Instructional material becomes available for material edits and design 3-5 days prior to start of program. Printing and assembly must be completed and delivered 24 hrs. prior to start of workshop."

- To provide the attached questions & answers

All other specifications, terms and conditions remain unchanged.

Patrick Johnson, **MBA**
Director of Procurement

Please **sign** below to acknowledge receipt of this Addendum and return with the proposal. Failure to return this Acknowledgement of Addendum may deem a proposal nonresponsive.

NOTE: ACKNOWLEDGEMENT OF RECEIPT OF BID ADDENDA WILL NOT BE ACCEPTED BY FACSIMILE OR E-MAIL.

Company Name _____ Authorized Signature _____

Date _____ Printed/Typed Signature _____

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Question	Answer
What color should binders be?	*Dark Blue*
Is tab printing required	*No*
Color or black and white?	*Color inserts on cover and spine*
Does the binder require a view pocket in front, and any spine printing?	*View pockets on front and spine*
Are there any other tab specifications?	*No*
Can you please provide further clarification for the design? Do you need the PPT file design fine-tuned (create color palette, font styles, clean up charts/graphs to make more appealing) and then printed on thumb drives?	*Yes, the slides require cleaning up for consistency, branding and ease of reading. Readability of projected images as well as readability of printed slides is a primary consideration. Generally, each page consists of two PPT slides but periodically a full page will be needed due to the complexity of a chart.* *A binder cover, cover sheet, and workshop outline are composed from scratch based on the course presentations that follow in each workbook.*
Or will PPT slides be placed into a page layout design with accompanying text?	*The attached work product example reflects a typical presentation within the course. The images are projected such that page formatting that might be found in textbooks would not be appropriate here. There is text on slides that requires reviewing and possible editing again for readability.*
Or will all of the content from the PPT files be redesigned into a book format?	*No*
Is it possible to provide an example of a previous PPT for reference? Seeing what is expected would help provide a more precise cost estimate.	*Yes, a typical example of workbook pages and slides are provided here. This example would be one presentation / section within the entire workbook.*
Can you provide an estimate of how many charts/graphs, schematics, etc. will be included in each workbook?	*The ratio of text to charts, graphs, schematics is approximately 40/60. Forty percent of the pages are text and sixty percent include graphics as shown in the example provided.*
Will the charts/graphs, schematics need to be recreated or just cleaned up?	*Generally, just formatted for readability.*
The RFQ requests only one sample. Is it possible to send more than one to show a large page count project as well as infographic/chart/graph capabilities?	*As we are interested in student workbook applications, please incorporate these capabilities in the single print exhibit and single thumb drive as requested in the RFQ.*
Are binders customized? Do they have a custom imprint on cover?	*No special imprint*
Does it matter if they are Round or D Ring?	*No*
Do the binders have inside pockets?	*Yes*

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If customized, does an inventory of binders need to be kept on hand? If so, how many?	*NA*

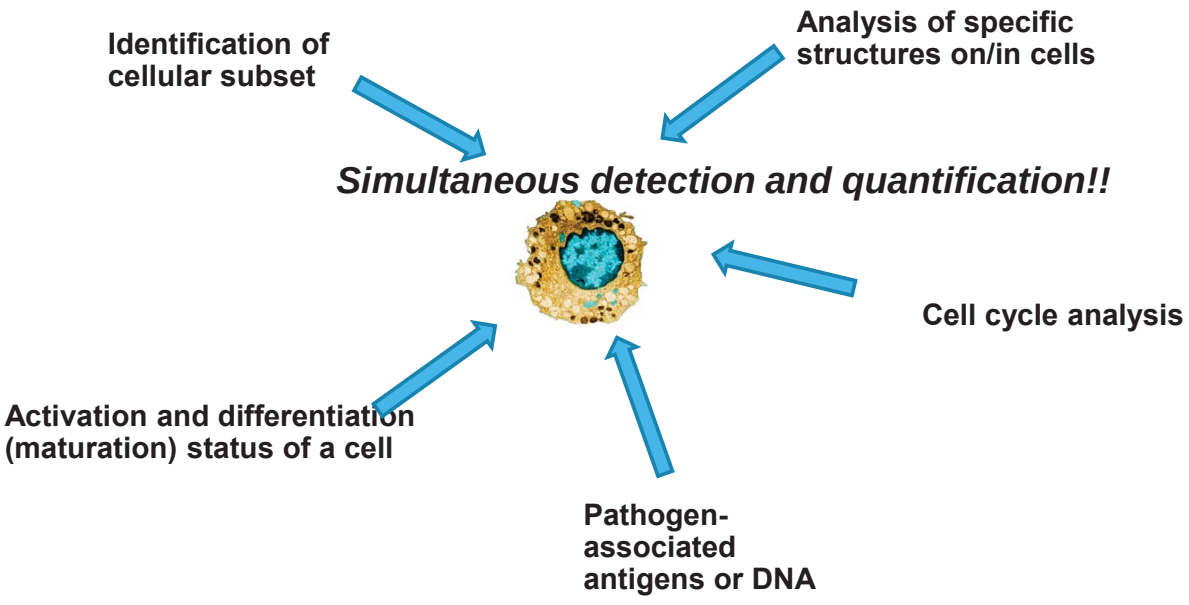
****************END OF QUESTIONS & ANSWERS******************

Introduction to Flow Cytometry

Dr. Elke S. Bergmann-Leitner

I. Introduction with Definition of Flow Cytometry
II. Comparison Flow Cytometry vs. Immunohistochemistry
III. General Setup of a Flow Cytometer
    1. Optical System
    2. Fluidic System
IV. Cell Sorting: Principles and Parameters
V. Parameter and Probes Useful for Flow Cytometric Analysis
    1. Measurement of Intrinsic Parameters
    2. Measurement of Extrinsic Parameters
VI. Data Analysis
    1. Types of Data Presentation: Dot Blot, Histogram, Contour Plot

Why Flow Cytometry?
Multi-parameter analysis!



Applications of Flow Cytometry

1. Cytology (cell cycle and cell growth, cell differentiation, necrotic and apoptotic events)
2. Molecular genetics

   Preparative tool for establishing chromosome specific DNA libraries
   - Mapping of chromosomes loci by spot-blot hybridization
   - Chromosome analysis and flow karyotyping
3. Oncology: e.g. analysis of cells from solid tumors
   - Cytokinetic studies to improve treatment protocols for diagnosis, prognosis
   - Simultaneous determination of DNA content and BrdU incorporation to measure the response of tumor cells to cancer therapeutic agents or radiation

Application - continued

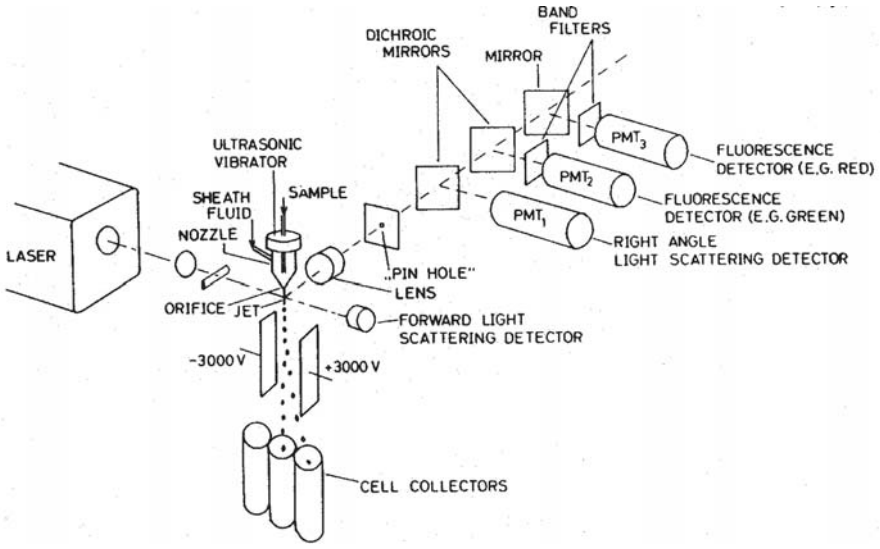
4. Microbiology
   - Detection and measurement of bacteria, protozoa or viruses in eukaryotic cells by DNA-, protein content, DNA base composition
   - Detection of toxins in the cytoplasma
   - Measuring higher membrane potential in parasitized cell
5. Animal breeding (analysis and sorting of sperms)
6. Clinical application
   - Oncology
   - Hematology (platelet counting, detection of specific leukocyte populations in the blood)

Application - continued

- Immunology
  - Measurement of T cell subpopulations (HIV)
  - Detection of antigen specific activated T cells
  - Intracellular staining for cytokines
  - Tetramer staining (compared to Elispot)
  - β-gal Fluorochrome (detection of transfected cells, detection of activated cells using β-gal-reporter gene)

7. Protein Biochemistry
   - Peptide binding to MHC class I or class II (T2 binding assays or fluorescent probes)
   - Staining for gene products inside the cell as alternative to immuno-precipitation and western blot (apoptosis, cell cycle and signaling)

Setup of a Flow Cytometer

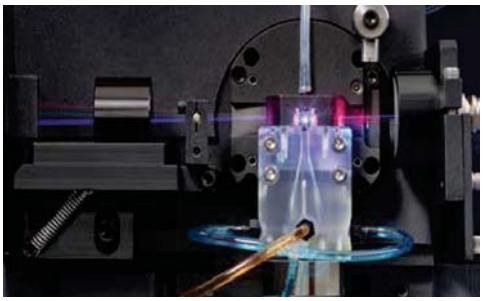


Laser-based flow cytometry with orthogonal configuration. Right-angle light scattering and two fluorescence components may be detected through microscope objective or equivalent lens perpendicular to the laser beam and water jet carrying cells.]Forward-angle scattering is measured by means of a solid-state detector close to the laser beam. A piezoelectric crystal causes the nozzle to vibrate at a frequency of the order of 40khz so that the water jet breaks into droplets for cell sorting by electrostatic deflection.

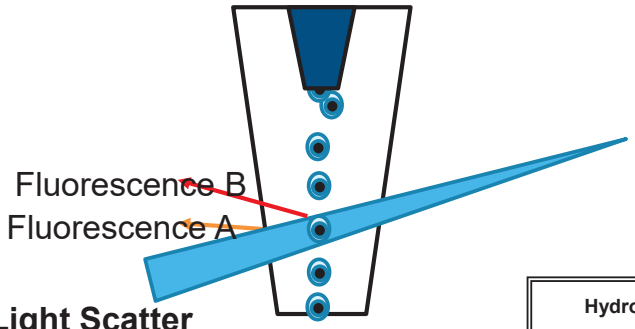
Setup of a Flow Cytometer: *Fluidic System*

Example of a Flow Cell – FACS Canto

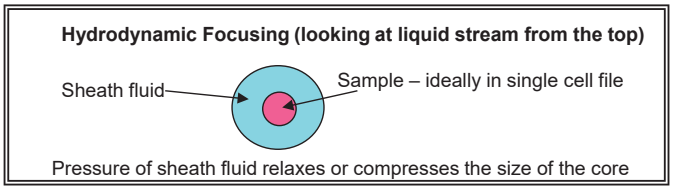
The flow cell – the heart of the flow cytometer



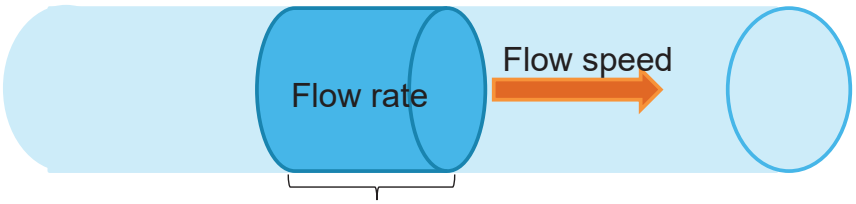
Events in the Flow Cell



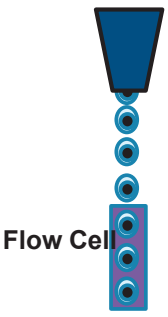
Light Scatter
Factor of cell size,
Cell shape, cytoplasmic granularity



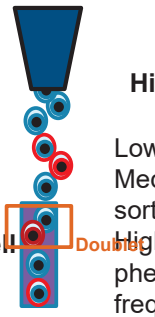
Flow Cytometric Definitions of Parameters Important for Proper Operations



Function of the differential pressure

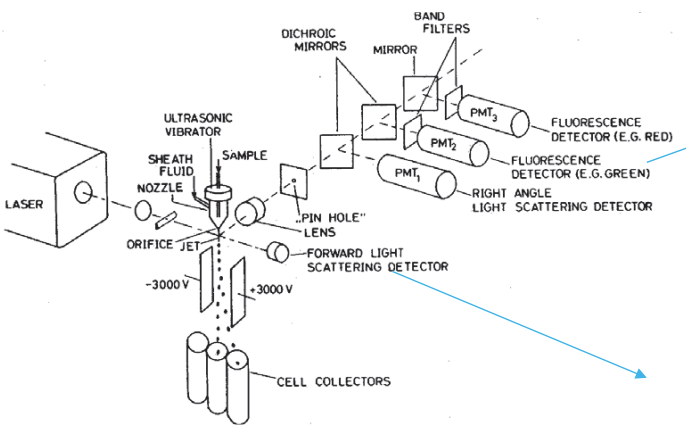


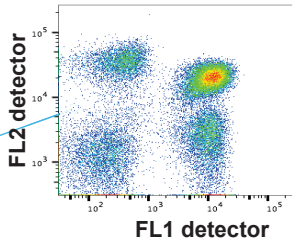
Low differential pressure
< 1.5 PSI
Higher resolution
More accurate doublet discrimination
Appropriate for cell cycle analysis
Preferable for cell sorting



High differential pressure
> 2 PSI
Lower resolution
Medium setting may be ok for sorting
High setting only acceptable for phenotyping cells when high frequency is expected

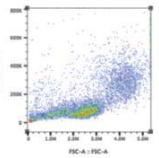
Setup of a Flow Cytometer: *Optical System*





Right Angle Light Detector
→ measures cell shape, cytoplasmic complexity

Incident Light Source

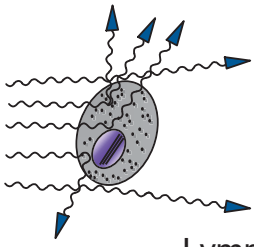


Forward Light Detector
→ measures cell size

Forward Scatter/Sideward Scatter

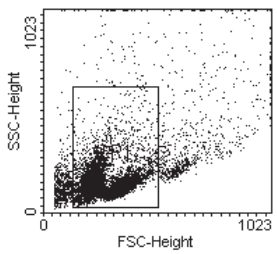
Right Angle Light Detector
→ measures cell shape, cytoplasmic complexity

Incident Light Source

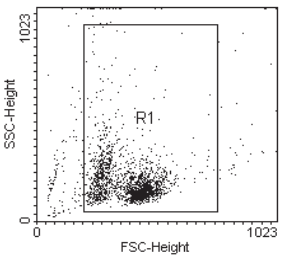


Forward Light Detector
→ measures cell size

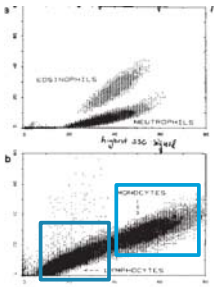
Splenocytes



Lymph node



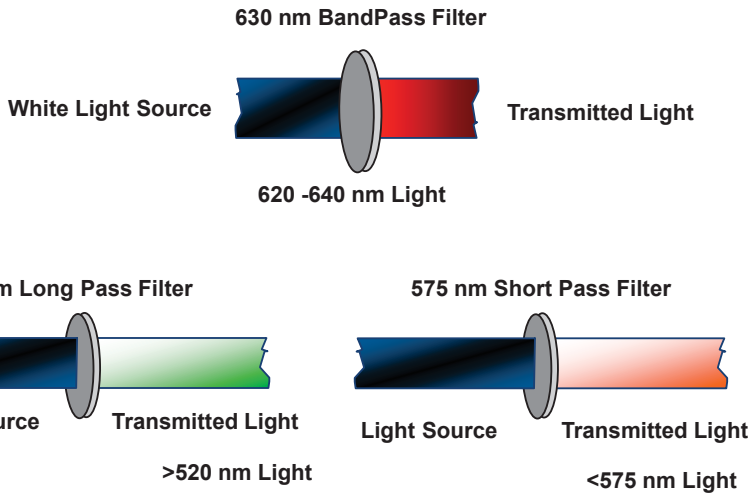
Peripheral Blood



Components of the Optical System: *Laser*

Laser	Excitation	Filters	Dyes (examples)
UV Laser	355 nm	379/28 - 820/60	BUV395 - BUV805
Violet Laser	405 nm	450/40 – 780/60	BV421 - BV786
Blue Laser	488 nm	530/30 – 780/60	FITC –PECy7
Yellow Laser	532 nm	575/26 - 780/60	PE - PECy7
Green Laser	561 nm	575/26 - 780/60	PE - PECy7
Red Laser	633 nm	660/20 - 780/60	APC – APC-H7

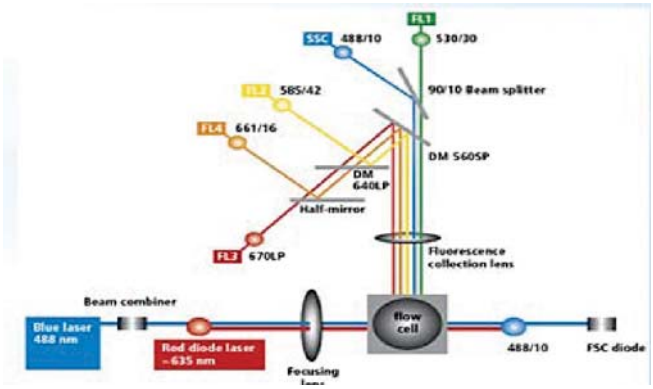
Components of the Optical System: *Standard Band Pass Filters*



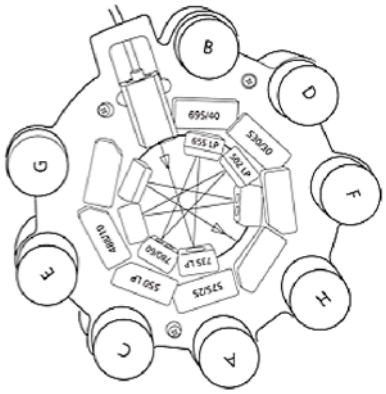
Making Space for More Colors:
How to send the fluorescent light to multiple detectors?

Arrangement of Photomultiplier Tubes (detectors)

Linear Arrangement → **Polygonal Arrangement**

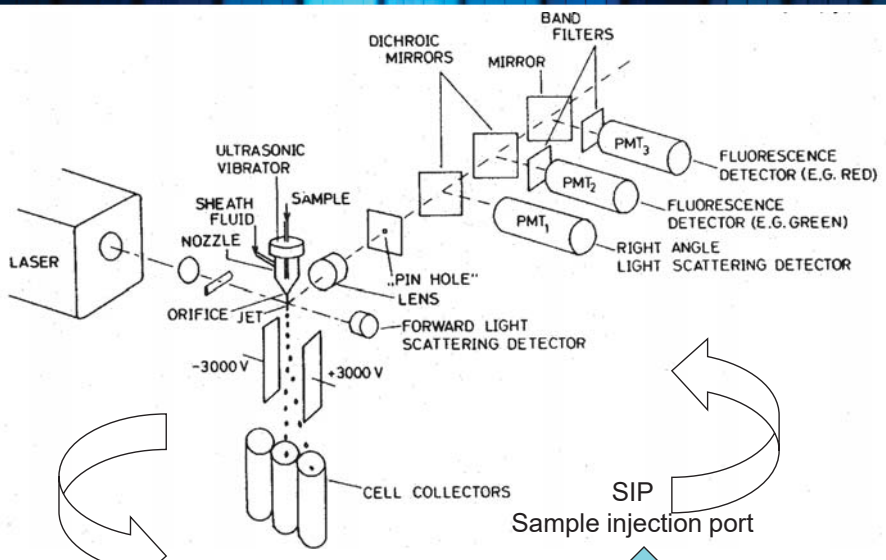


Fluorescent light from the cell



http://www.bdbiosciences.com/br/instruments/facscantoresearch/features/optics.jsp

Fluidic System

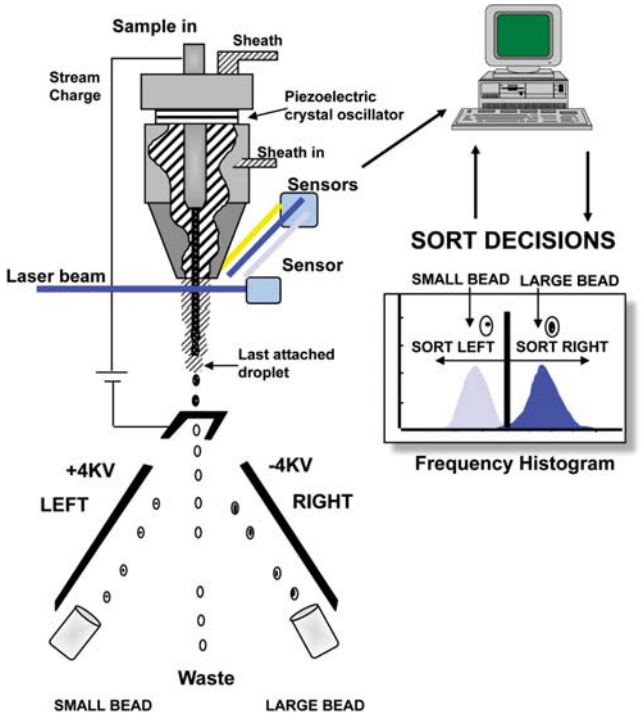


SIP
Sample injection port

Waste Container

Pressurized
Sheath Fluid Container

Flow Sorting



Cellular Parameters for Flow Analysis: *What do you measure?*

	Structural	Functional
Intrinsic	Cell size Cell shape Cytoplasmic granularity Pigment content	Redox state
Extrinsic	DNA content DNA base composition Chromatin structure RNA content Total protein content, basic protein Surface sugars	Membrane integrity Membrane permeability Enzyme activity Endocytosis Surface charge Extra-, intracellular receptors DNA synthesis Cytoplasmic/mitochondrial membrane potential Intracellular Ca^{2+}

Choosing a Fluorochrome

- Three crucial pieces of information needed when choosing a fluorochrome:
- (1) What are the specs of the laser? → Excitation wavelength
- (2) What are the specs of the dichroic mirrors,
- band pass filters of the flow cytometer? → what are the cut-ons/offs?
- (3) are the fluorochromes considered compatible?

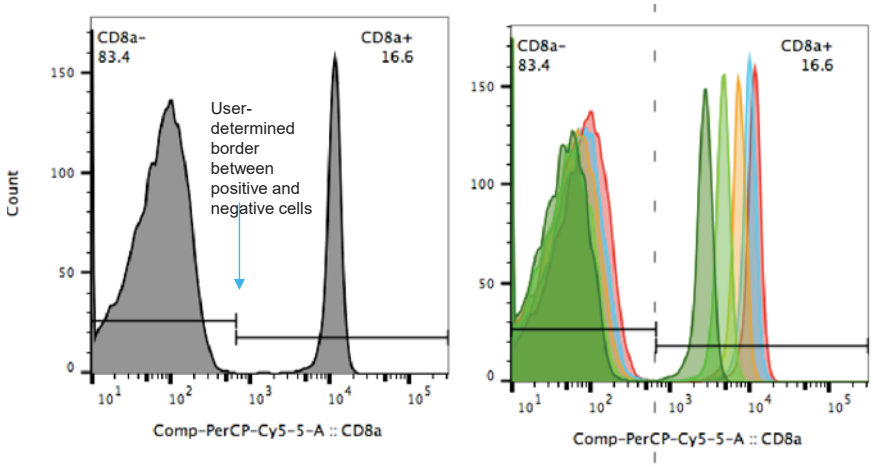
Choosing a Fluorochrome

Fluorchrome	Absorption maximum	Emission maximum
FITC	490 nm	520 nm
TRITC	554 nm	573 nm
Texas Red	596 nm	620 nm
Phycoerythrin-R	480-582 nm	570 nm
XRITC	580 nm	600 nm
Allophycocyanine	650 nm	680 nm
PerCP	490 nm	650 nm
CyChrome (PE-Cy5)	490 nm	650 nm

Three crucial pieces of information needed when choosing a fluorochrome:
(1) What are the specs of the laser? → Excitation wavelength
(2) What are the specs of the dichroic mirrors, band pass filters of the flow cytometer?
→ what are the cut-ons/offs?
(3) are the fluorochromes compatible with each other?

Data Presentation Styles: *Histograms*

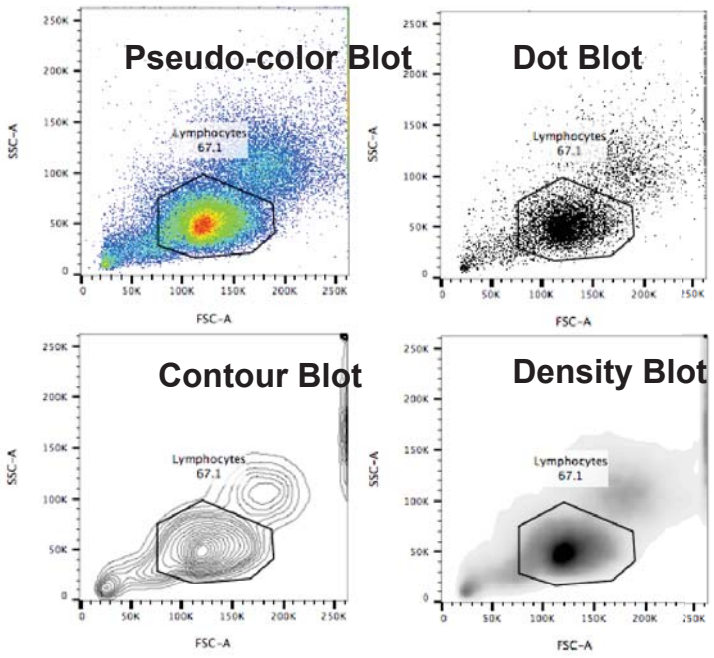
Histogram display → showing one parameter at a time

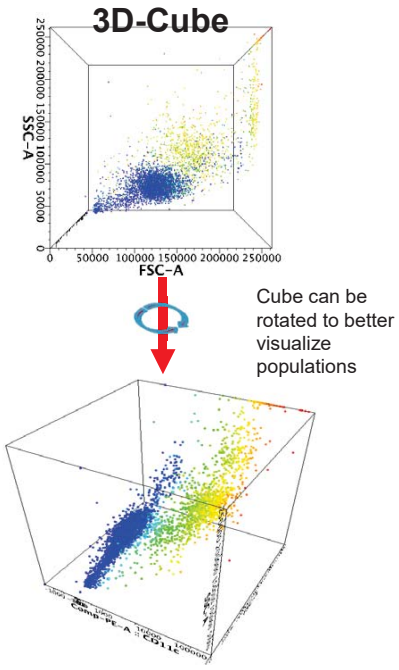


Histogram analysis for determining the percentage of cells that express a specific marker and those that do not

Histogram overlay for side-by-side comparison of multiple samples

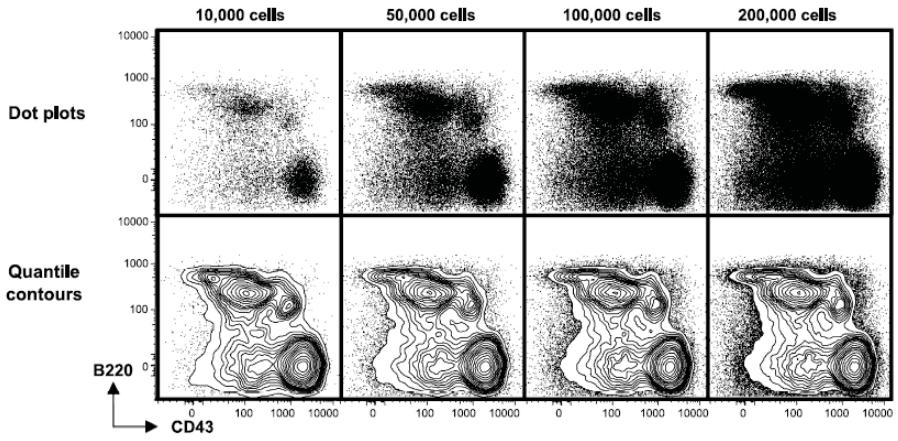
Data Presentation Styles: *2-3 parameters*





Cube can be rotated to better visualize populations

Data Presentation – Which one to choose



Dense cell clusters cannot be resolved in dot blots → require alternative display modes such as contour blot

Application of Flow Cytometry in Various Areas of Basic Research

I. Cytology
   1. Cell cycle analysis
   2. Cell differentiation
   3. Necrotic and apoptotic events

II. Molecular Genetics
   1. Preparative tool for establishing chromosome specific DNA libraries
   2. Mapping of chromosome loci by spot-blot hybridization
   3. Chromosome analysis and flow karyotyping

III. Oncology: e.g. analysis of cells from solid tumors

1. Cytokinetic studies to improve treatment protocols for diagnosis, prognosis
2. Simultaneous determination of DNA content and BrdU incorporation to measure the response of tumor cells to cancer therapeutic agents or radiation

IV. Microbiology

1. Detection of bacteria, protozoa or viruses in eukaryotic cells by DNA-, protein-content, DNA base composition
2. Detection of toxins in the cytoplasm
3. Measurement of higher membrane potential in parasitzed cells

V. Animal breeding

1. Analysis and sorting of sperms

Classic Karyotyping

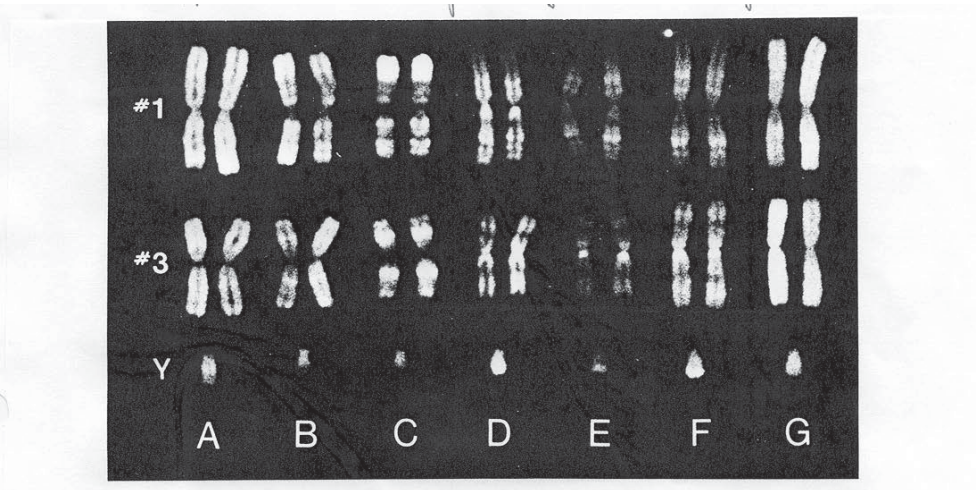


Fig. 24. Dependence of fluorescence contrast enhancement by DNA-binding counterstains on relative dye binding specificities and spectral overlap. Shown are human 1,3 and Y chromosomes from different cells of the same individual. Chromosomes in columns A and B were stained only with chromomycin A_3 at 500 μM (A) or 4 μM (B), in 0.14 M pH 6.8 phosphate buffer plus $MgCl_2$. Chromosomes in column C were stained with 500 μM chromomycin A_3 followed by 100 μM methyl green, the latter in 0.15 M NaCl/0.005 M Hepes, pH 7.0. Chromosomes in column D were stained with 0.4 μM 33258 Hoechst, in 0.14 M NaCl/0.004 M KCl/0.01 M phosphate, pH 7.0, while those in columns E, F, and G were stained with 0.4 μM 33258 Hoechst, followed by 500 μM chromomycin A_3. Fluorescence from doubly stained chromosomes was due to chromomycin A_3 in C and G and to 33258 Hoechst in E and F. The mounting medium in E was pH 7.5 McIlvaine's buffer. In all other cases, glycerol, which stabilizes chromomycin A_3 fluorescence, was used. Chromomycin A_3 staining solutions contained equimolar amounts of the dye and magnesium [365].

Staining of Chromosomes

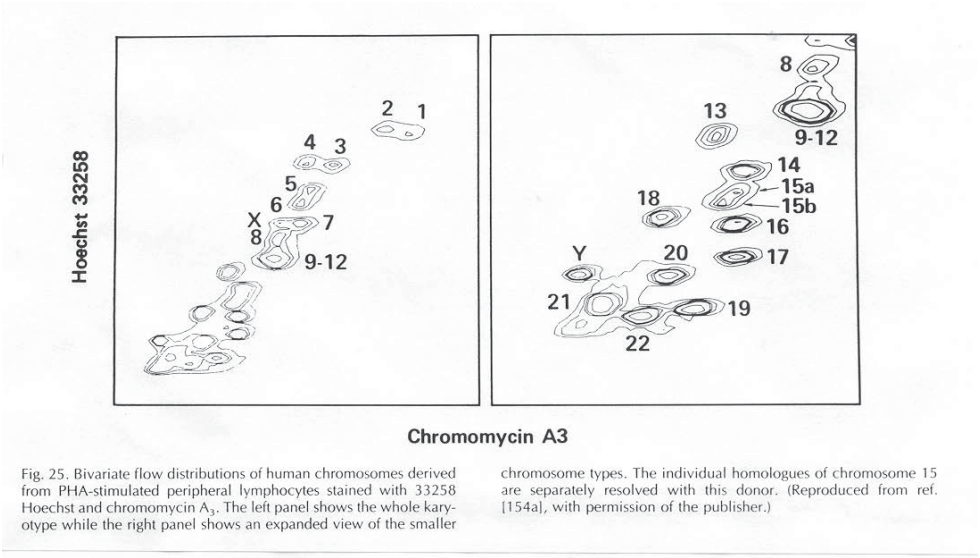


Fig. 25. Bivariate flow distributions of human chromosomes derived from PHA-stimulated peripheral lymphocytes stained with 33258 Hoechst and chromomycin A_3. The left panel shows the whole karyotype while the right panel shows an expanded view of the smaller chromosome types. The individual homologues of chromosome 15 are separately resolved with this donor. (Reproduced from ref. [154a], with permission of the publisher.)

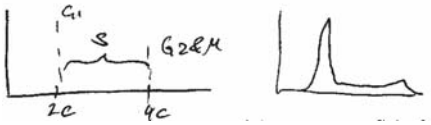
Application: Cell Cycle Analysis

DNA, RNA, content, cell size and BrdU incorporation are valuable parameters to define the position of the cells in cell cycle.

DNA Content

Non-replicating cells in G0, G1 have same DNA content (2c)

Show typical distribution in histogram.



Standardization with chicken RBC (35%DNA of human 2c cells) and rainbow trout RBC (80%) to calibrate the system.

DNA Base Composition

Some dyes have preference for either A-T or G-C rich DNA
Quinacrine has same affinity for either one, but binding to A-T results in brighter signal than G-C.
Hoeschst 33258: A-T
Chromomycin: G-C

Allows karyotyping (see transparent)

Cell Cycle Analysis Cont'd

Chromatin Structure

Fix cells and treat with RNAse (most of the dyes would also bind to RNA). Partial denaturation of DNA by acid or heat. Subsequent staining with AO will then reveal chromatin structure:
AO binding to native DNA will result in emission of given wavelength, binding to denatured DNA will result in shift of wavelength (higher). Sensitivity of DNA denaturation correlates with the chromatin condensation; highest in mitosis and quiescent cells, most resistant DNA in late G1, early S-phase

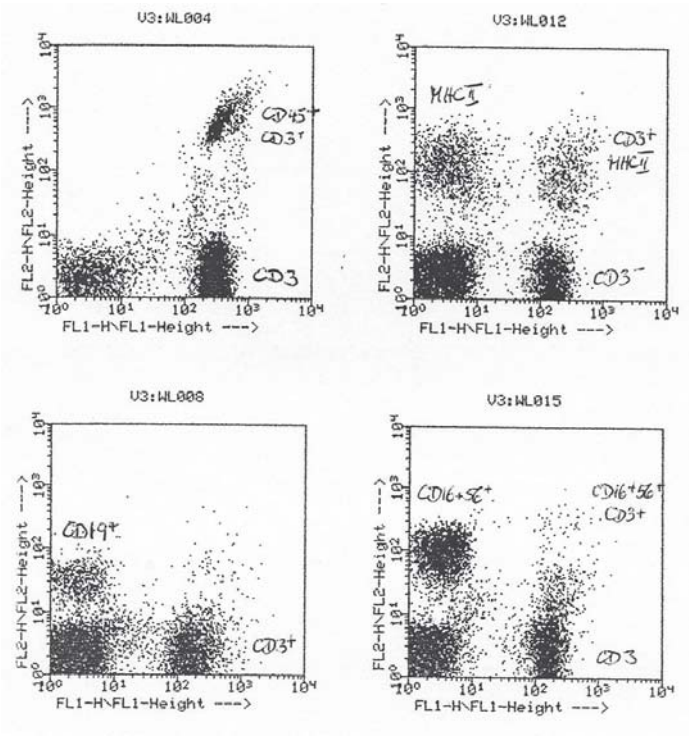
AO/acid denaturation gives more information about the cell's progress through cell cycle than DNA content measurement alone.

Application of Flow Cytometry in Various Areas of Clinical Research

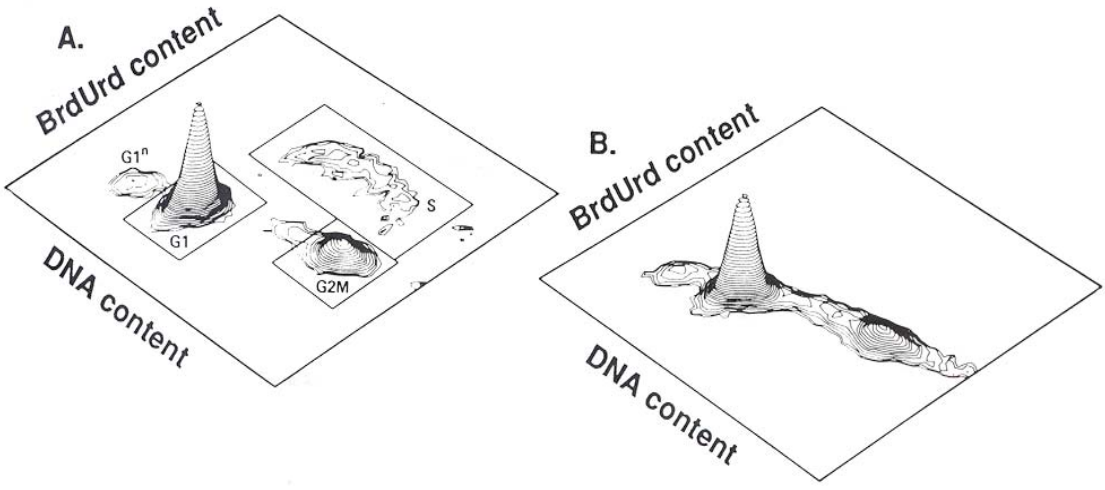
VI. Clinical Application

1. Oncology
2. Hematology (platelet counting, detection of particular leukocyte populations in the blood
3. Immunology
   - Measurement of T cell subpopulations (e.g. HIV)
   - Detection of Ag-specific, activated T cells
   - Intracellular staining for growth factors
   - Evaluation of responses to vaccination: ELIspot *vs*. tetramer-staining

Phenotyping of Human Peripheral Blood Mononuclear Cells

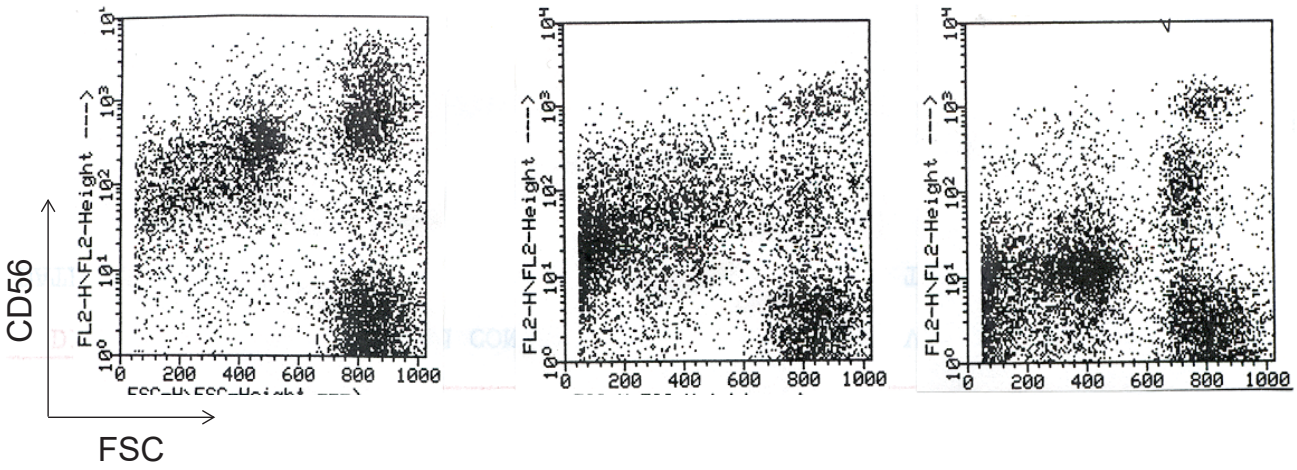


Detection of Proliferating Cells



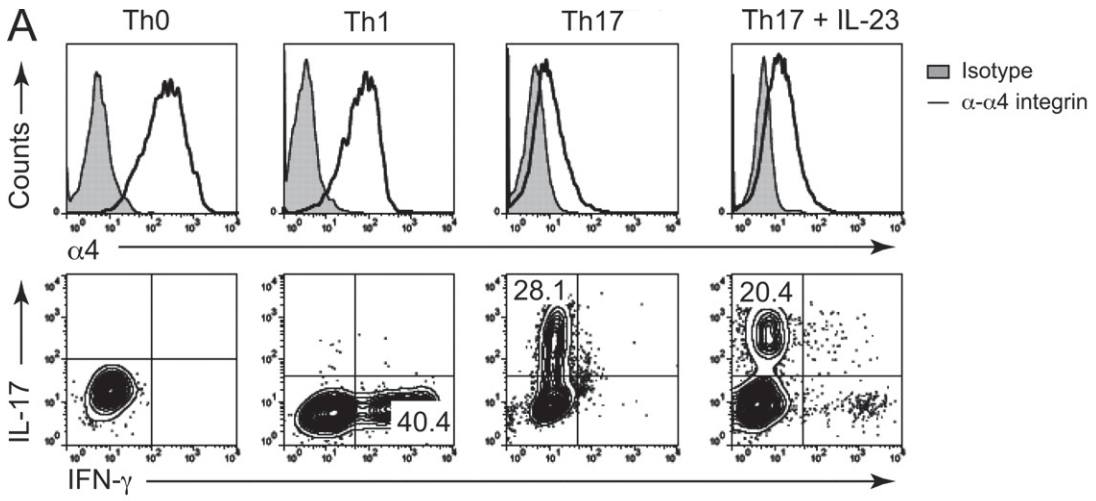
Bivariate distribution (contour plot) of DNA content versus incorporated BrdUrd for human, All cells grown *in vitro*. B: The same type of plot as for panel A, after the cells were treated with ara-c, showing the loss of cells from S-phase.

Detection of Leukocyte Subsets

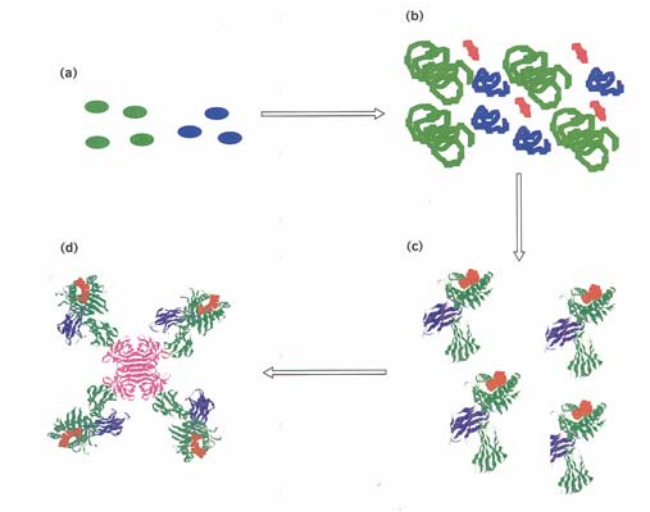


Intracellular Staining for Cytokines

…determines the frequency of cells producing a specific factor (alternate to ELISpots)



Tetramer-staining



(a) Heavy chain is modified by substitution of transmembrane and cytosolic regions with biotinylated target sequence for enzyme BirA

(b) Purification of recombinant proteins form inclusion bodies of E.coli: refolding step *in vitro* and addition of epitope peptide ==> isolation of refolded complexes by FPLC

(c) Loaded complexes are biotinylated at C-terminal end of heavy chain by BirA. Removal of free biotin by FPLC and ion exchange chromatography.

(d) Fluorescent Streptavidin A is added to induce tetramer formation

Other approach: biotinylate all lysine residues of β2m.

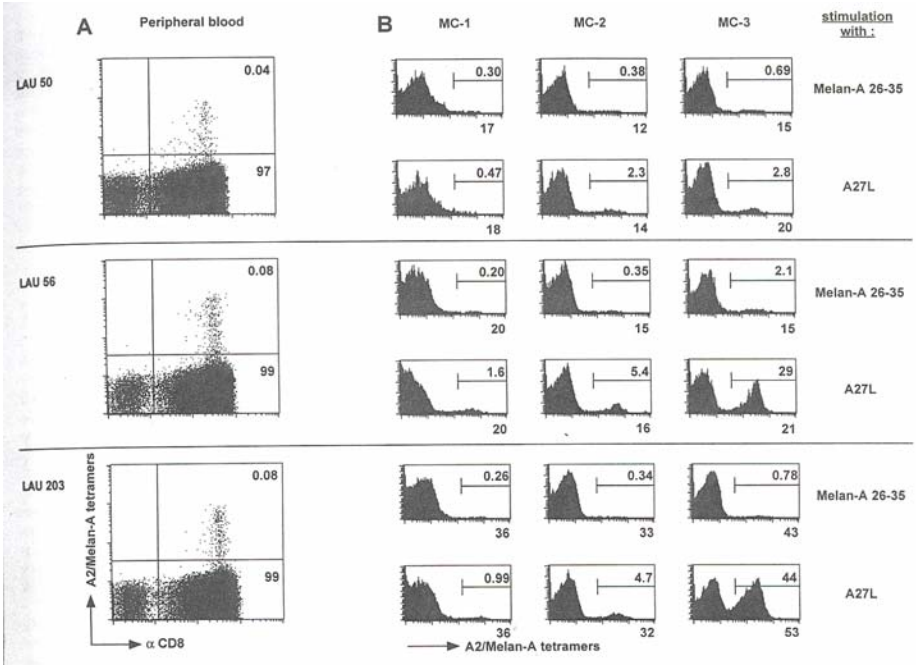
Protocol for Staining of Ag-specific T Cells with Tetramers

Protocol for Staining of Ag-specific T Cells with Tetramers

- Stain cells in PBS+5%serum with 1mg/ml tetramer for 20min at 4ºC.
- Add anti-CD8 or other mAb (10mg/ml) for 30min at 4ºC.
- Wash cells and analyze in FACS

Gating of Populations Can Increase the Sensitivity of the Analysis

Results based on all PBMC Results gated on CD8⁺ T cells



Application of Flow Cytometry in Various Areas of Biochemistry

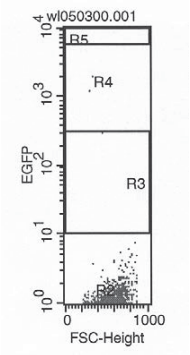
VII. Protein Chemistry

1. Peptide binding to MHC class I or class II (T2 assay, fluorescent peptides)

VIII. Flow Cytometry as an Alternative to Western Blot

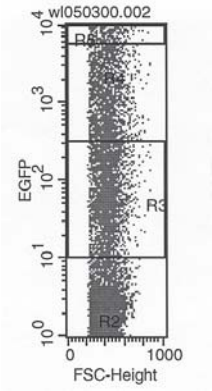
IX. Monitoring the Efficiency of Transfection

Transient Transfection of Cells with a Plasmid



Control (non transfected)

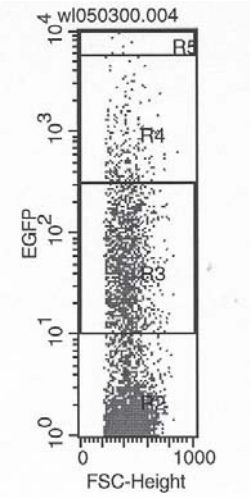
Region	Events	% Gated	% Total	X Mean	X Geo Mean
R1	7971	80.78	72.87	476.66	471.07
R2	9864	99.97	90.18	453.03	443.20
R3	1	0.01	0.01	459.00	459.00
R4	2	0.02	0.02	320.50	320.07
R5	0	0.00	0.00	***	***



Good transfection

Region	Events	% Gated	% Total	X Mean	X Geo Mean
R1	5864	61.64	52.10	464.58	459.60
R2	4893	51.43	43.47	399.93	388.17
R3	2586	27.18	22.98	422.20	408.21
R4	1809	19.01	16.07	431.06	414.40
R5	248	2.61	2.20	441.19	423.34

Transient Transfection of Cells with a Plasmid, Cont'd

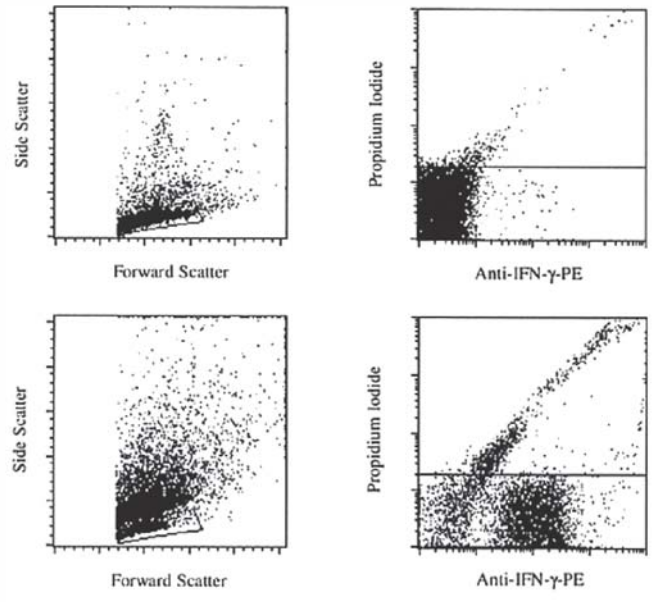


Bad transfection

Region	Events	% Gated	% Total	X Mean	X Geo Mean
R1	5558	58.73	51.65	459.29	454.65
R2	7838	82.83	72.84	395.70	383.46
R3	1401	14.81	13.02	419.14	404.49
R4	230	2.43	2.14	415.95	397.87
R5	7	0.07	0.07	384.71	377.23

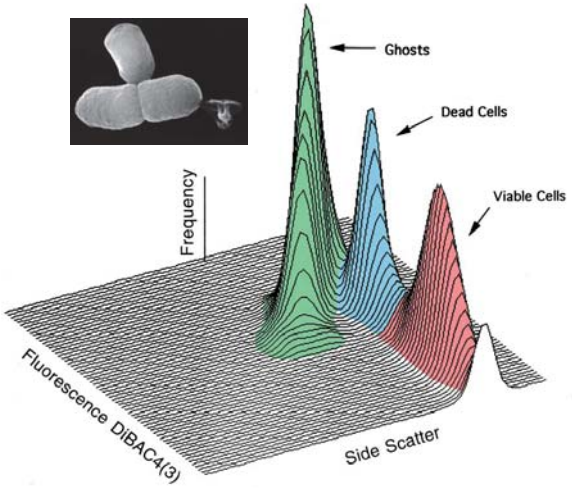
Live/Dead Distinction Using FACS Analysis

Eukaryotic cells: Live/dead distinction based on FSC/SSC characteristics or DNA stains such as PI, 7-AAD etc.



Live/Dead Distinction Using FACS Analysis, Cont'd

Prokaryotic cells: Live/dead distinction based on SSC vs. DIBAC staining



Introduction to Flow Cytometry

Dr. Elke S. Bergmann-Leitner

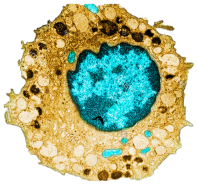
I. Introduction with Definition of Flow Cytometry
II. Comparison Flow Cytometry vs. Immunohistochemistry
III. General Setup of a Flow Cytometer
    1. Optical System
    2. Fluidic System
IV. Cell Sorting: Principles and Parameters
V. Parameter and Probes Useful for Flow Cytometric Analysis
    1. Measurement of Intrinsic Parameters
    2. Measurement of Extrinsic Parameters
VI. Data Analysis
    1. Types of Data Presentation: Dot Blot, Histogram, Contour Plot

Why Flow Cytometry?
Multi-parameter analysis!

Identification of cellular subset

Analysis of specific structures on/in cells

Simultaneous detection and quantification!!



Cell cycle analysis

Activation and differentiation (maturation) status of a cell

Pathogen-associated antigens or DNA

Applications of Flow Cytometry

1. Cytology (cell cycle and cell growth, cell differentiation, necrotic and apoptotic events)
2. Molecular genetics

   Preparative tool for establishing chromosome specific DNA libraries

   - Mapping of chromosomes loci by spot-blot hybridization
   - Chromosome analysis and flow karyotyping
3. Oncology: e.g. analysis of cells from solid tumors
   - Cytokinetic studies to improve treatment protocols for diagnosis, prognosis
   - Simultaneous determination of DNA content and BrdU incorporation to measure the response of tumor cells to cancer therapeutic agents or radiation

Application - continued

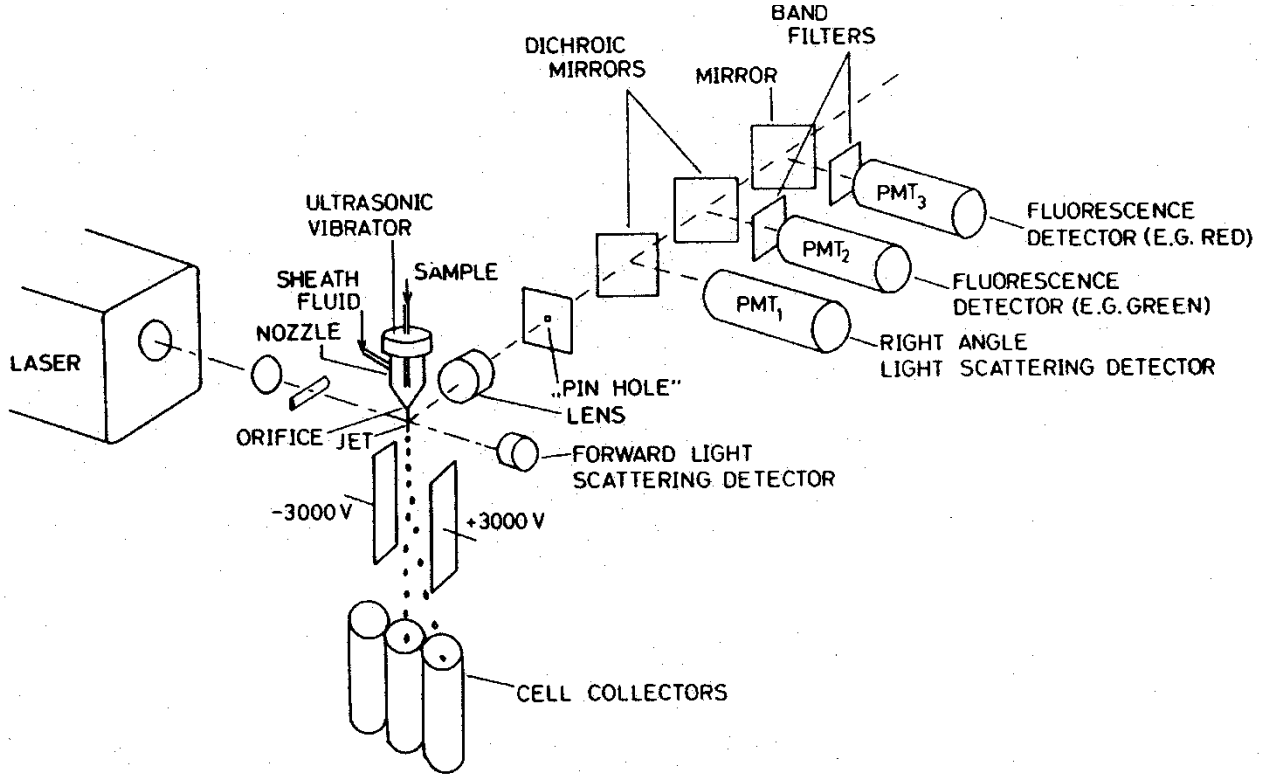
4. Microbiology
   - Detection and measurement of bacteria, protozoa or viruses in eukaryotic cells by DNA-, protein content, DNA base composition
   - Detection of toxins in the cytoplasma
   - Measuring higher membrane potential in parasitized cell
5. Animal breeding (analysis and sorting of sperms)
6. Clinical application
   - Oncology
   - Hematology (platelet counting, detection of specific leukocyte populations in the blood)

Application - continued

- Immunology
  - Measurement of T cell subpopulations (HIV)
  - Detection of antigen specific activated T cells
  - Intracellular staining for cytokines
  - Tetramer staining (compared to Elispot)
  - β-gal Fluorochrome (detection of transfected cells, detection of activated cells using β-gal-reporter gene)

7. Protein Biochemistry
   - Peptide binding to MHC class I or class II (T2 binding assays or fluorescent probes)
   - Staining for gene products inside the cell as alternative to immuno-precipitation and western blot (apoptosis, cell cycle and signaling)

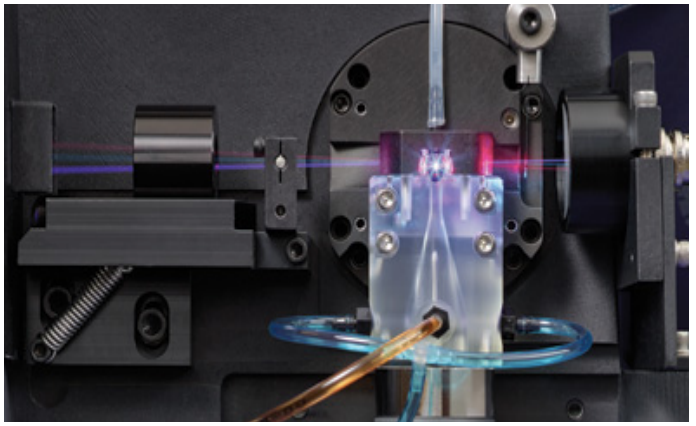
Setup of a Flow Cytometer



Laser-based flow cytometry with orthogonal configuration. Right-angle light scattering and two fluorescence components may be detected through microscope objective or equivalent lens perpendicular to the laser beam and water jet carrying cells.]Forward-angle scattering is measured by means of a solid-state detector close to the laser beam. A piezoelectric crystal causes the nozzle to vibrate at a frequency of the order of 40khz so that the water jet breaks into droplets for cell sorting by electrostatic deflection.

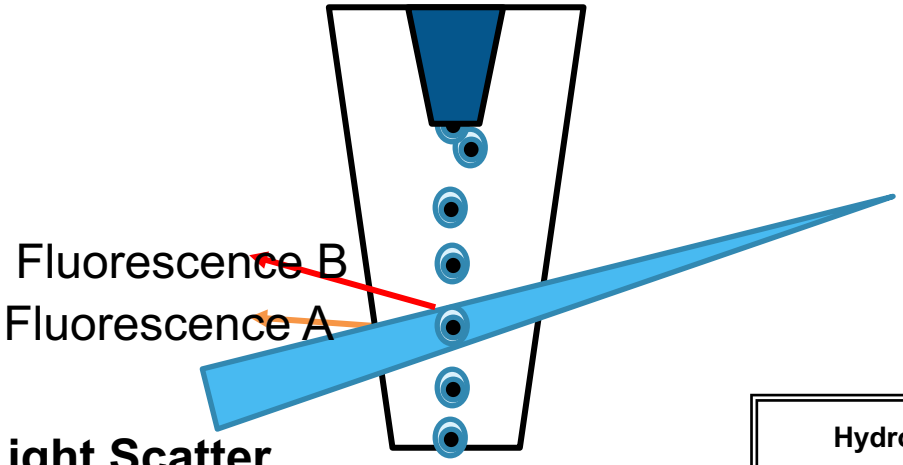
Setup of a Flow Cytometer: *Fluidic System*

Example of a Flow Cell – FACS Canto

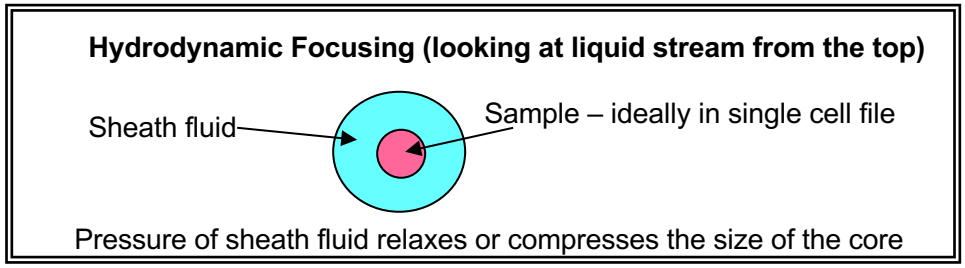


The flow cell – the heart of the flow cytometer

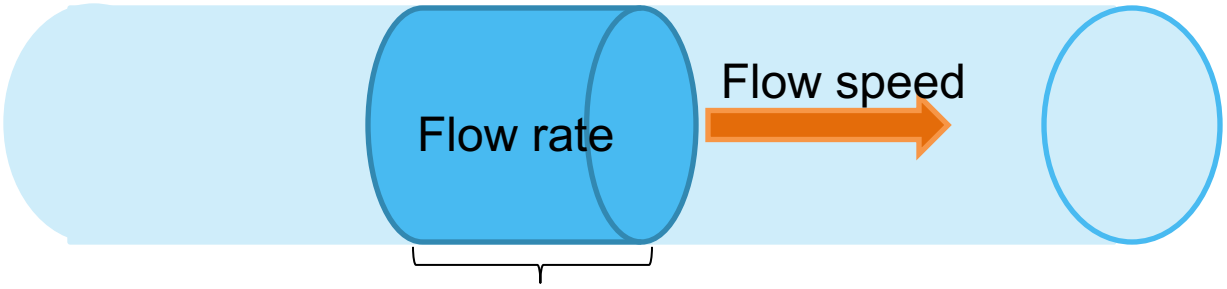
Events in the Flow Cell



Light Scatter
Factor of cell size,
Cell shape, cytoplasmic granularity

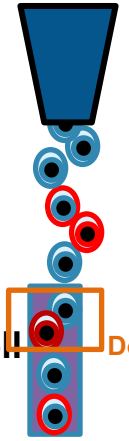


Flow Cytometric Definitions of Parameters Important for Proper Operations



Low differential pressure
< 1.5 PSI

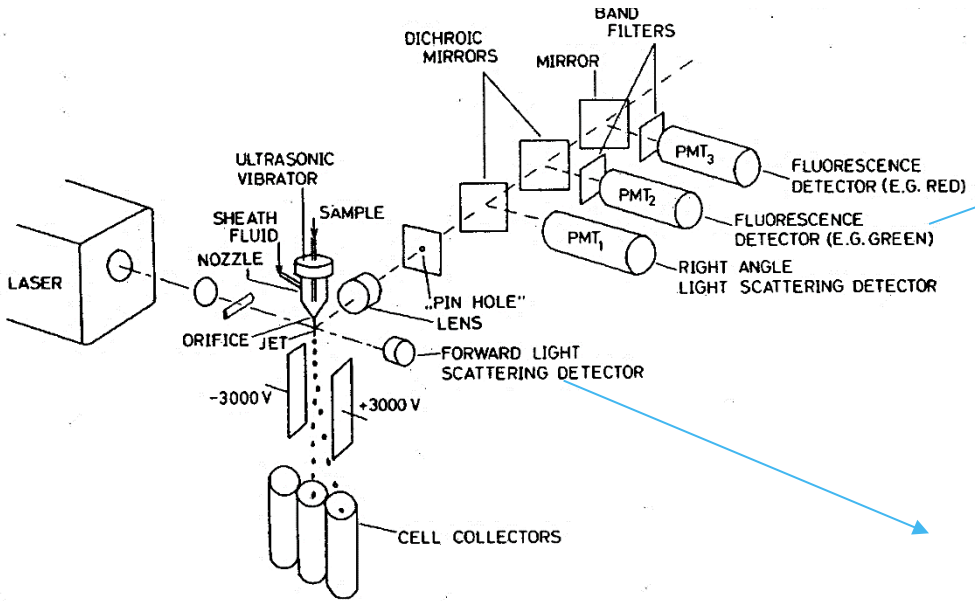
Higher resolution
More accurate doublet discrimination
Appropriate for cell cycle analysis
Preferable for cell sorting

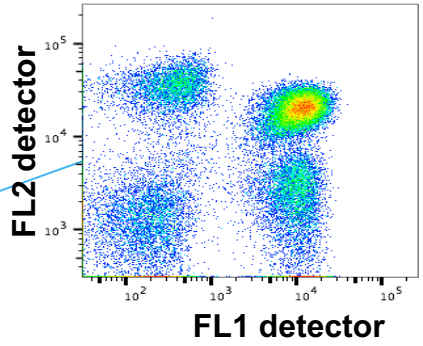


High differential pressure
> 2 PSI

Lower resolution
Medium setting may be ok for sorting
High setting only acceptable for phenotyping cells when high frequency is expected

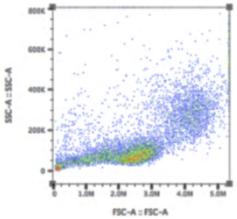
Setup of a Flow Cytometer: *Optical System*





Right Angle Light Detector
→ measures cell shape, cytoplasmic complexity

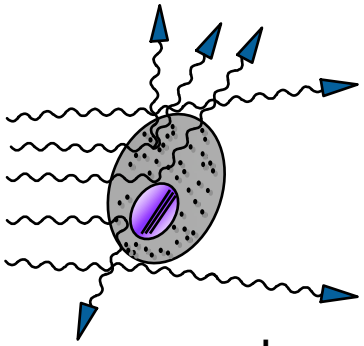
Incident Light Source



Forward Light Detector
→ measures cell size

Forward Scatter/Sideward Scatter

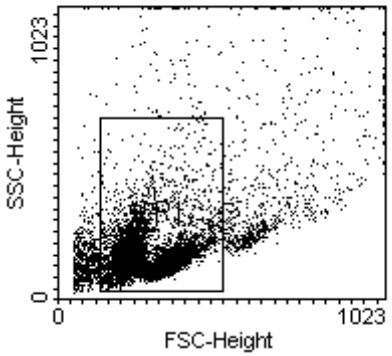
Right Angle Light Detector
→ measures cell shape, cytoplasmic complexity



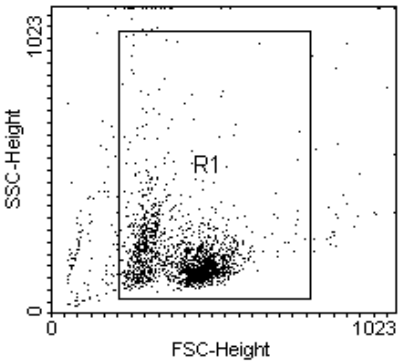
Incident Light Source

Forward Light Detector
→ measures cell size

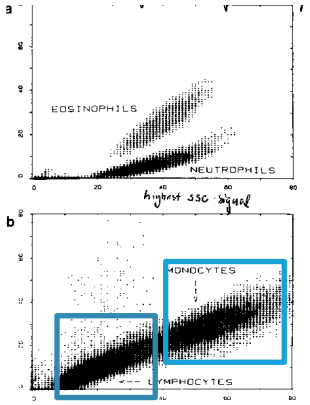
Splenocytes



Lymph node



Peripheral Blood



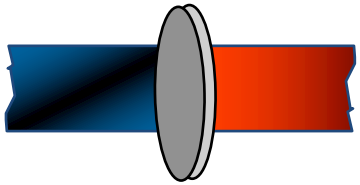
Components of the Optical System: *Laser*

Laser	Excitation	Filters	Dyes (examples)
UV Laser	355 nm	379/28 - 820/60	BUV395 - BUV805
Violet Laser	405 nm	450/40 – 780/60	BV421 - BV786
Blue Laser	488 nm	530/30 – 780/60	FITC –PECy7
Yellow Laser	532 nm	575/26 - 780/60	PE - PECy7
Green Laser	561 nm	575/26 - 780/60	PE - PECy7
Red Laser	633 nm	660/20 - 780/60	APC – APC-H7

Components of the Optical System:
Standard Band Pass Filters

630 nm BandPass Filter

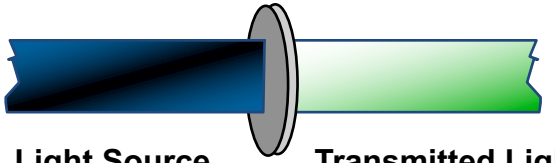
White Light Source



Transmitted Light

620 -640 nm Light

520 nm Long Pass Filter

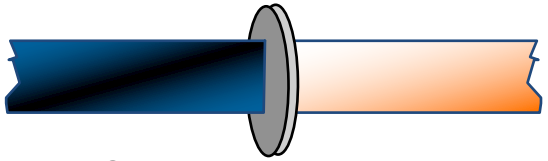


Light Source

Transmitted Light

>520 nm Light

575 nm Short Pass Filter



Light Source

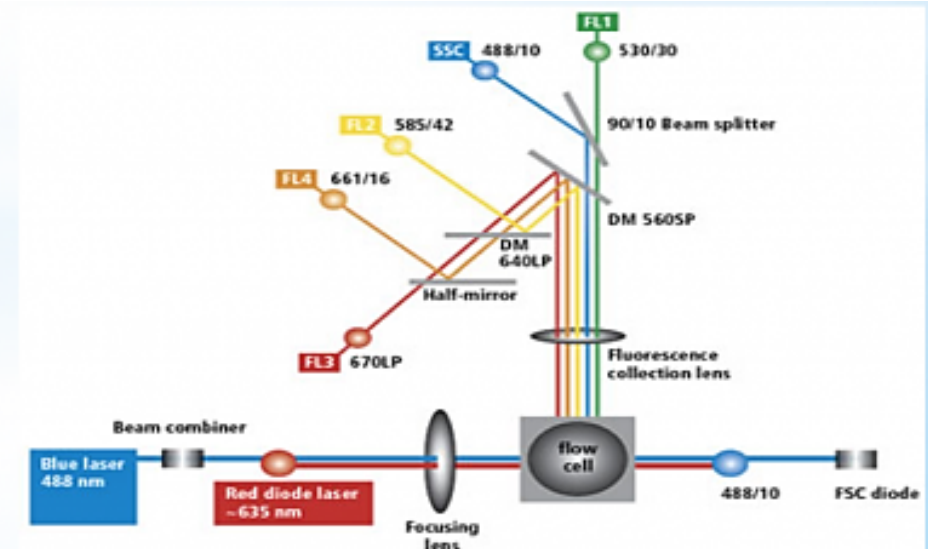
Transmitted Light

<575 nm Light

Making Space for More Colors:
How to send the fluorescent light to multiple detectors?

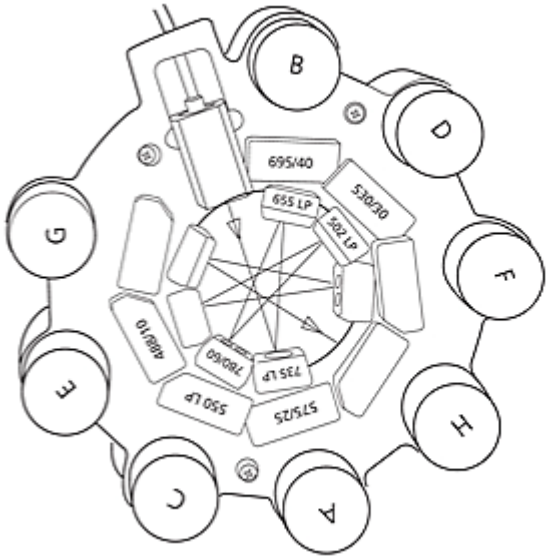
Arrangement of Photomultiplier Tubes (detectors)

Linear Arrangement



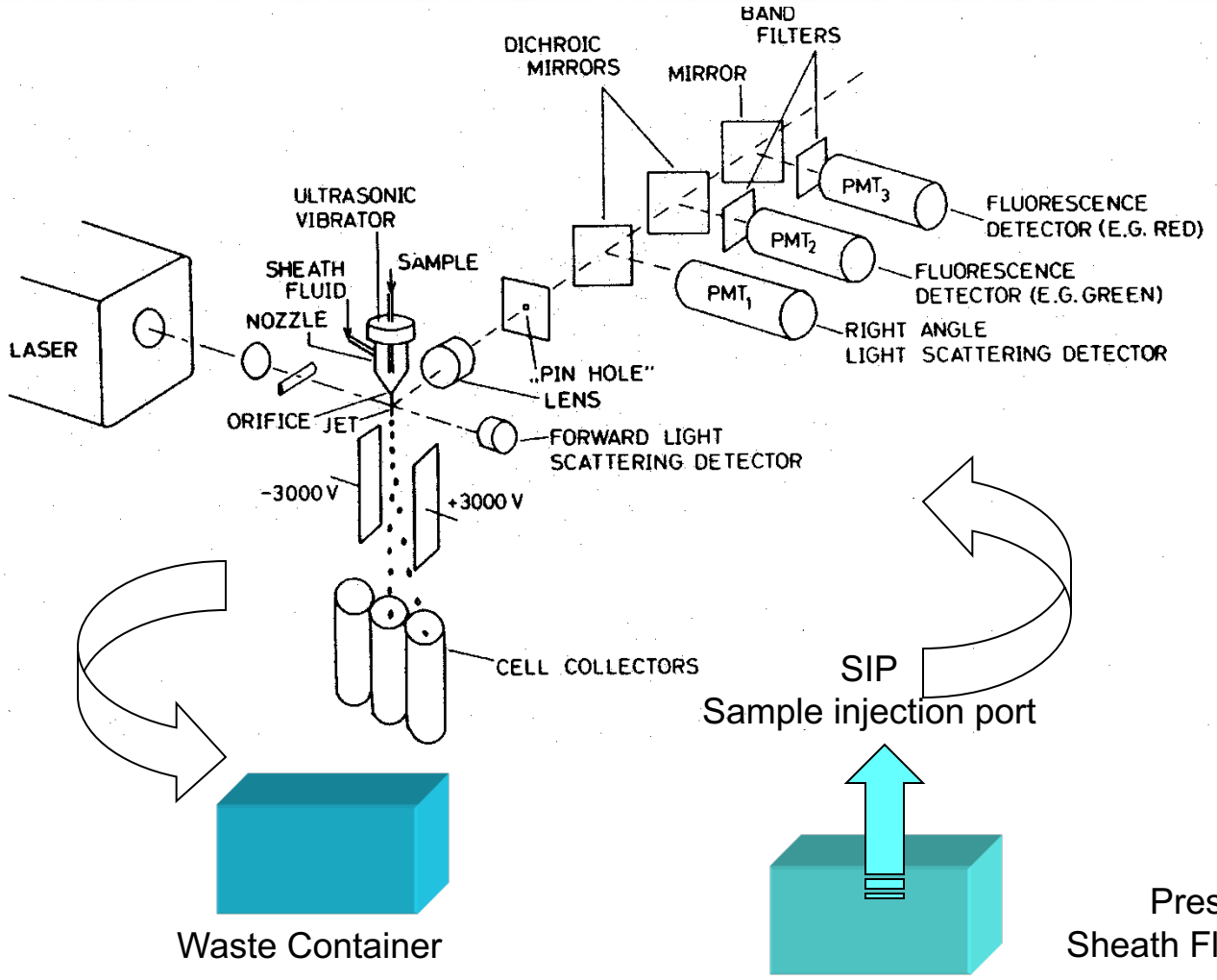
Polygonal Arrangement

Fluorescent light from the cell

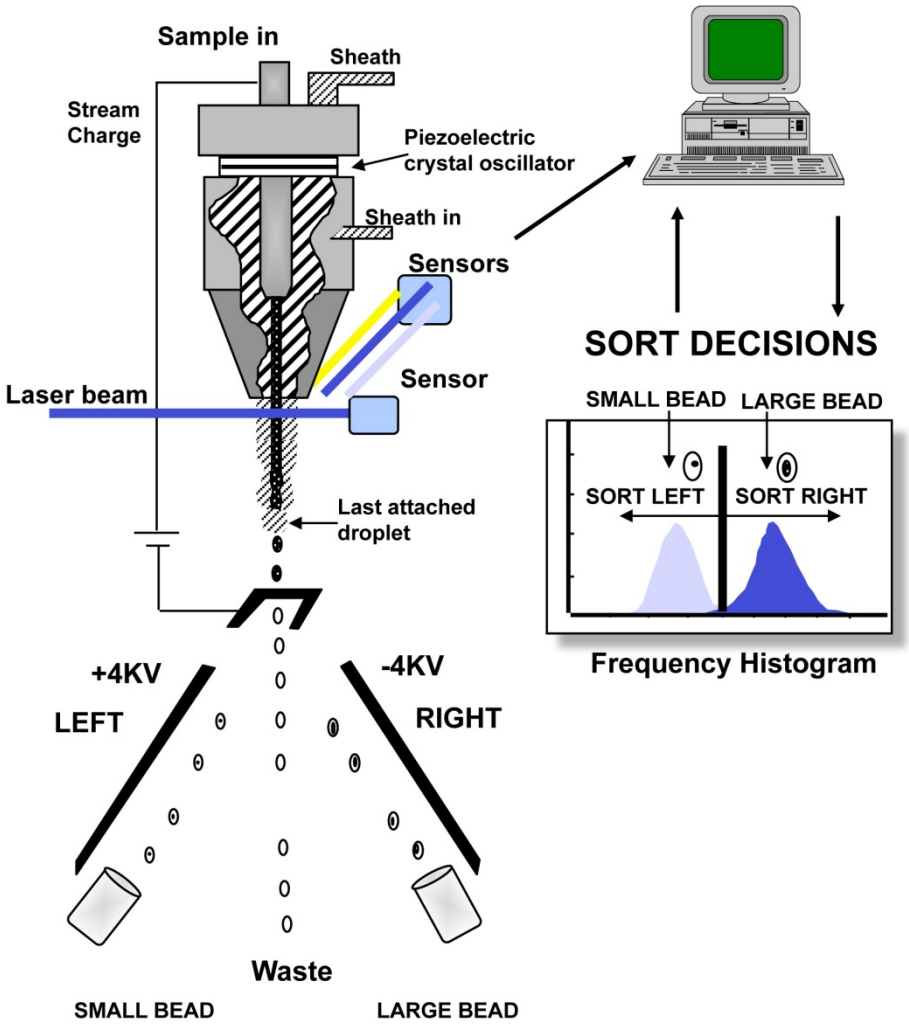


http://www.bdbiosciences.com/br/instruments/facscantoresearch/features/optics.jsp

Fluidic System



Flow Sorting



Cellular Parameters for Flow Analysis: *What do you measure?*

	Structural	**Functional**
Intrinsic	Cell size Cell shape Cytoplasmic granularity Pigment content	Redox state
Extrinsic	DNA content DNA base composition Chromatin structure RNA content Total protein content, basic protein Surface sugars	Membrane integrity Membrane permeability Enzyme activity Endocytosis Surface charge Extra-, intracellular receptors DNA synthesis Cytoplasmic/mitochondrial membrane potential Intracellular Ca^{2+}

Choosing a Fluorochrome

- Three crucial pieces of information needed when choosing a fluorochrome:
- (1) What are the specs of the laser? → Excitation wavelength
- (2) What are the specs of the dichroic mirrors,
- band pass filters of the flow cytometer? → what are the cut-ons/offs?
- (3) are the fluorochromes considered compatible?

Choosing a Fluorochrome

Fluorchrome	Absorption maximum	Emission maximum
FITC	490 nm	520 nm
TRITC	554 nm	573 nm
Texas Red	596 nm	620 nm
Phycoerythrin-R	480-582 nm	570 nm
XRITC	580 nm	600 nm
Allophycocyanine	650 nm	680 nm
PerCP	490 nm	650 nm
CyChrome (PE-Cy5)	490 nm	650 nm

Three crucial pieces of information needed when choosing a fluorochrome:

(1) What are the specs of the laser? → Excitation wavelength

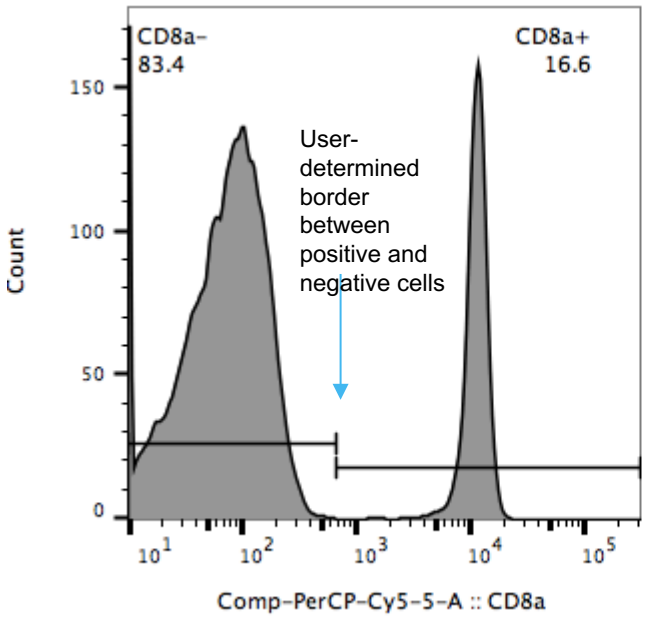
(2) What are the specs of the dichroic mirrors, band pass filters of the flow cytometer?

→ what are the cut-ons/offs?

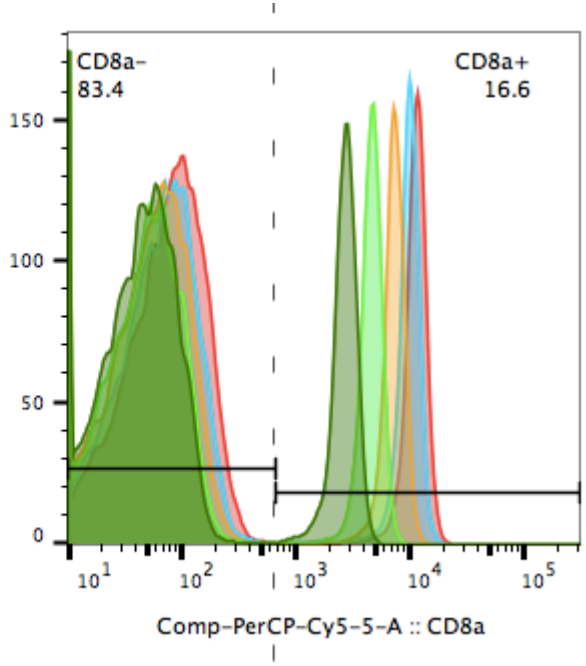
(3) are the fluorochromes compatible with each other?

Data Presentation Styles: *Histograms*

Histogram display → showing one parameter at a time

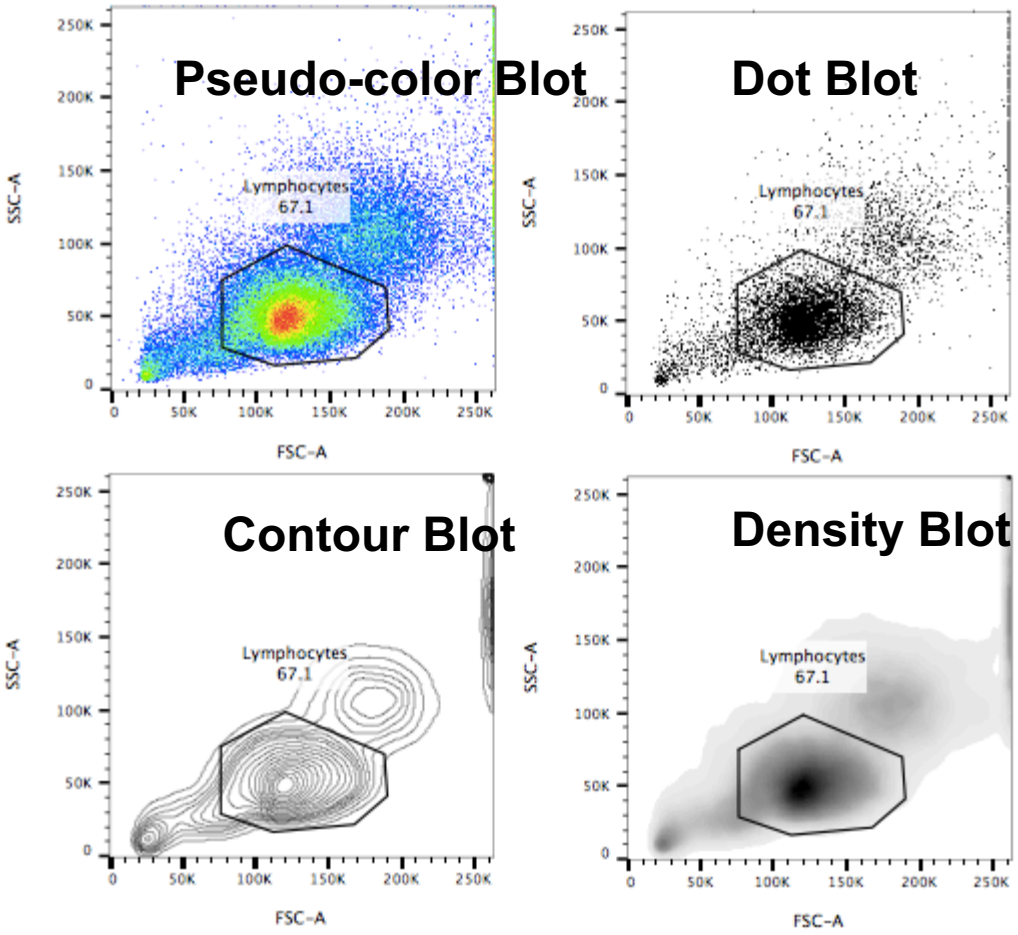


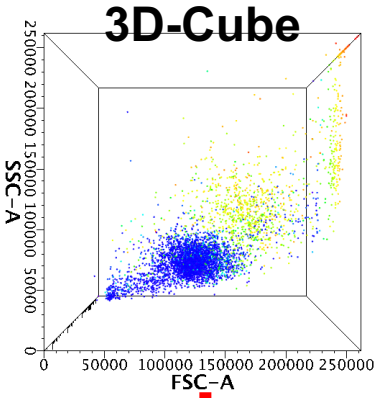
Histogram analysis for determining the percentage of cells that express a specific marker and those that do not



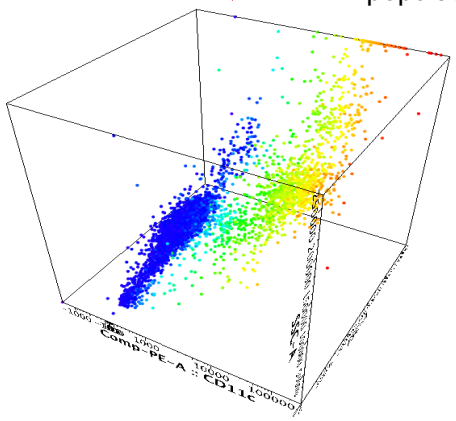
Histogram overlay for side-by-side comparison of multiple samples

Data Presentation Styles: *2-3 parameters*

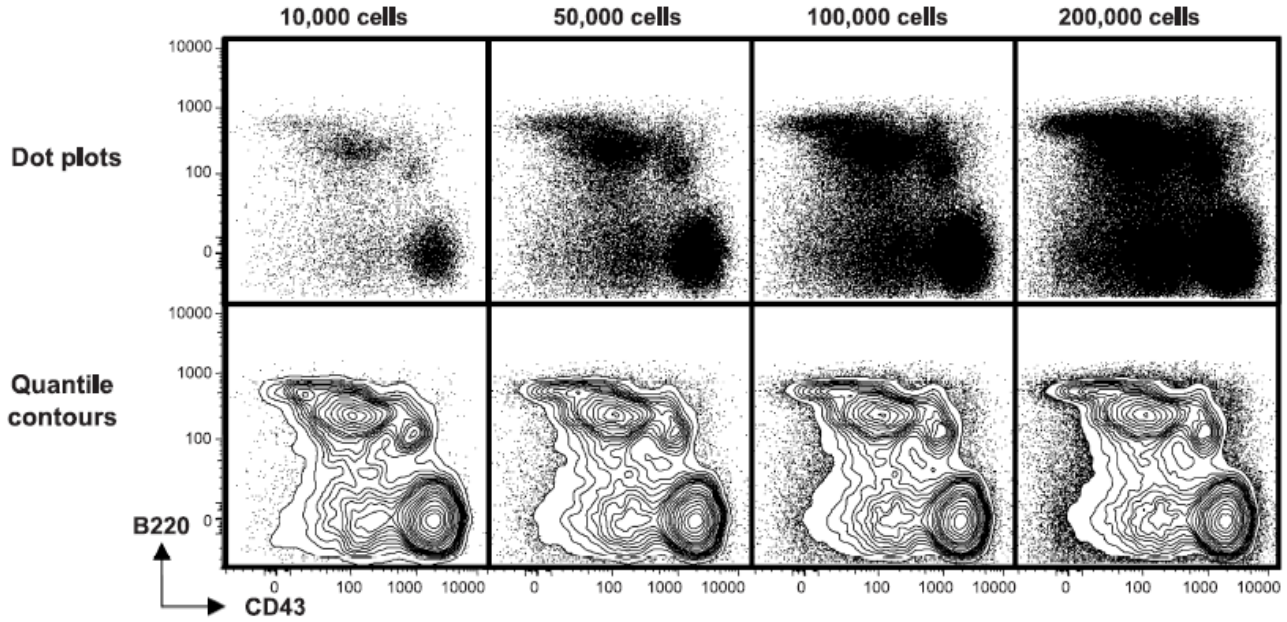




Cube can be rotated to better visualize populations



Data Presentation – Which one to choose



Dense cell clusters cannot be resolved in dot blots → require alternative display modes such as contour blot

Application of Flow Cytometry in Various Areas of Basic Research

I. Cytology
   1. Cell cycle analysis
   2. Cell differentiation
   3. Necrotic and apoptotic events

II. Molecular Genetics
   1. Preparative tool for establishing chromosome specific DNA libraries
   2. Mapping of chromosome loci by spot-blot hybridization
   3. Chromosome analysis and flow karyotyping

III. Oncology: e.g. analysis of cells from solid tumors
    1. Cytokinetic studies to improve treatment protocols for diagnosis, prognosis
    2. Simultaneous determination of DNA content and BrdU incorporation to measure the response of tumor cells to cancer therapeutic agents or radiation

IV. Microbiology
    1. Detection of bacteria, protozoa or viruses in eukaryotic cells by DNA-, protein-content, DNA base composition
    2. Detection of toxins in the cytoplasm
    3. Measurement of higher membrane potential in parasitzed cells

V. Animal breeding
    1. Analysis and sorting of sperms

Classic Karyotyping

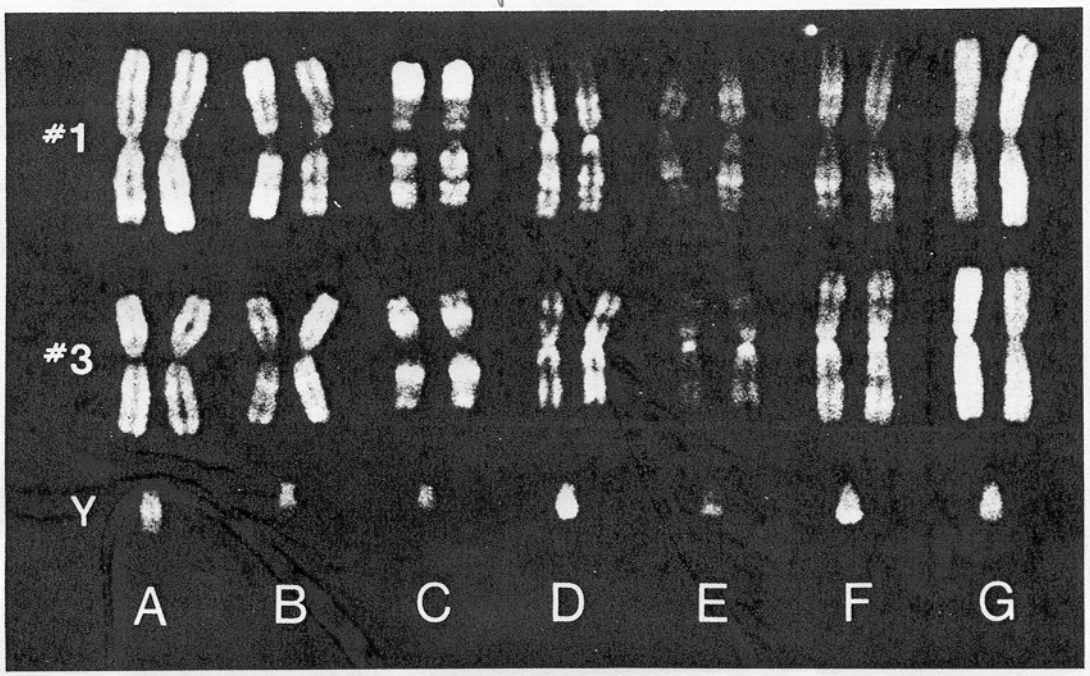


Fig. 24. Dependence of fluorescence contrast enhancement by DNA-binding counterstains on relative dye binding specificities and spectral overlap. Shown are human 1,3 and Y chromosomes from different cells of the same individual. Chromosomes in columns A and B were stained only with chromomycin A_3 at 500 μM (A) or 4 μM (B), in 0.14 M pH 6.8 phosphate buffer plus $MgCl_2$. Chromosomes in column C were stained with 500 μM chromomycin A_3 followed by 100 μM methyl green, the latter in 0.15 M NaCl/0.005 M Hepes, pH 7.0. Chromosomes in column D were stained with 0.4 μM 33258 Hoechst, in 0.14 M NaCl/0.004 M KCl/0.01 M phosphate, pH 7.0, while those in columns E, F, and G were stained with 0.4 μM 33258 Hoechst, followed by 500 μM chromomycin A_3. Fluorescence from doubly stained chromosomes was due to chromomycin A_3 in C and G and to 33258 Hoechst in E and F. The mounting medium in E was pH 7.5 McIlvaine's buffer. In all other cases, glycerol, which stabilizes chromomycin A_3 fluorescence, was used. Chromomycin A_3 staining solutions contained equimolar amounts of the dye and magnesium [365].

Staining of Chromosomes

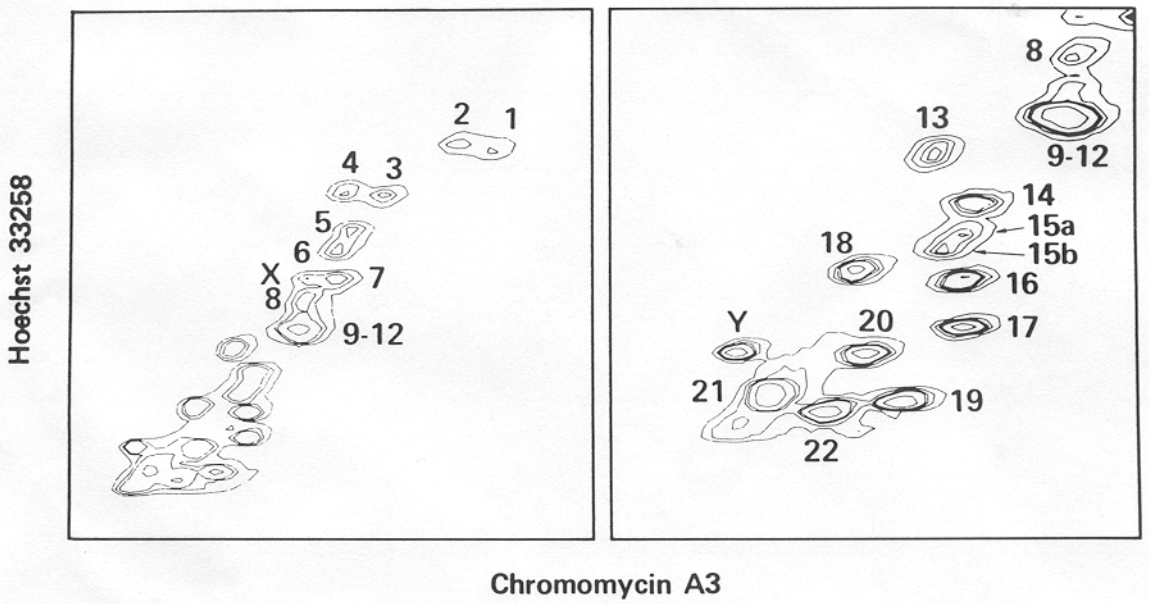


Fig. 25. Bivariate flow distributions of human chromosomes derived from PHA-stimulated peripheral lymphocytes stained with 33258 Hoechst and chromomycin A_3. The left panel shows the whole karyotype while the right panel shows an expanded view of the smaller chromosome types. The individual homologues of chromosome 15 are separately resolved with this donor. (Reproduced from ref. [154a], with permission of the publisher.)

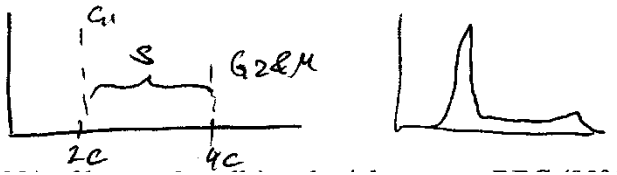
Application: Cell Cycle Analysis

DNA, RNA, content, cell size and BrdU incorporation are valuable parameters to define the position of the cells in cell cycle.

DNA Content

Non-replicating cells in G0, G1 have same DNA content (2c)

Show typical distribution in histogram.



Standardization with chicken RBC (35%DNA of human 2c cells) and rainbow trout RBC (80%) to calibrate the system.

DNA Base Composition

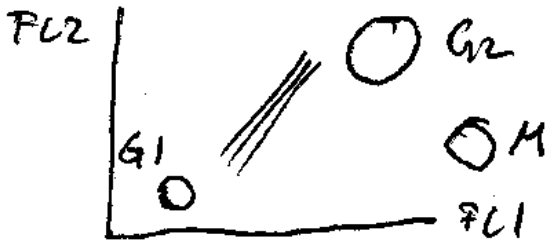
Some dyes have preference for either A-T or G-C rich DNA
Quinacrine has same affinity for either one, but binding to A-T results in brighter signal than G-C.
Hoeschst 33258: A-T
Chromomycin: G-C

Allows karyotyping (see transparent)

Cell Cycle Analysis Cont'd

Chromatin Structure

Fix cells and treat with RNAse (most of the dyes would also bind to RNA). Partial denaturation of DNA by acid or heat. Subsequent staining with AO will then reveal chromatin structure:
AO binding to native DNA will result in emission of given wavelength, binding to denatured DNA will result in shift of wavelength (higher). Sensitivity of DNA denaturation correlates with the chromatin condensation; highest in mitosis and quiescent cells, most resistant DNA in late G1, early S-phase



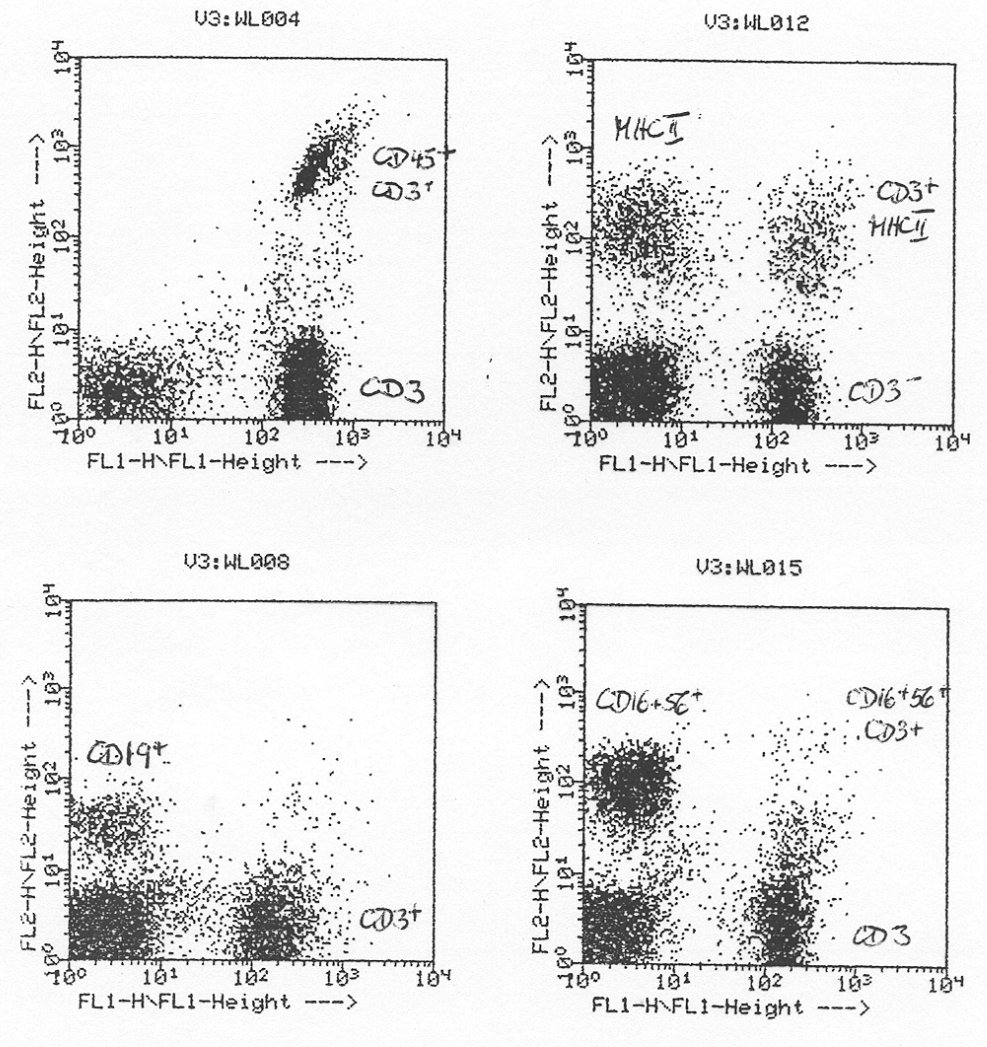
AO/acid denaturation gives more information about the cell's progress through cell cycle than DNA content measurement alone.

Application of Flow Cytometry in Various Areas of Clinical Research

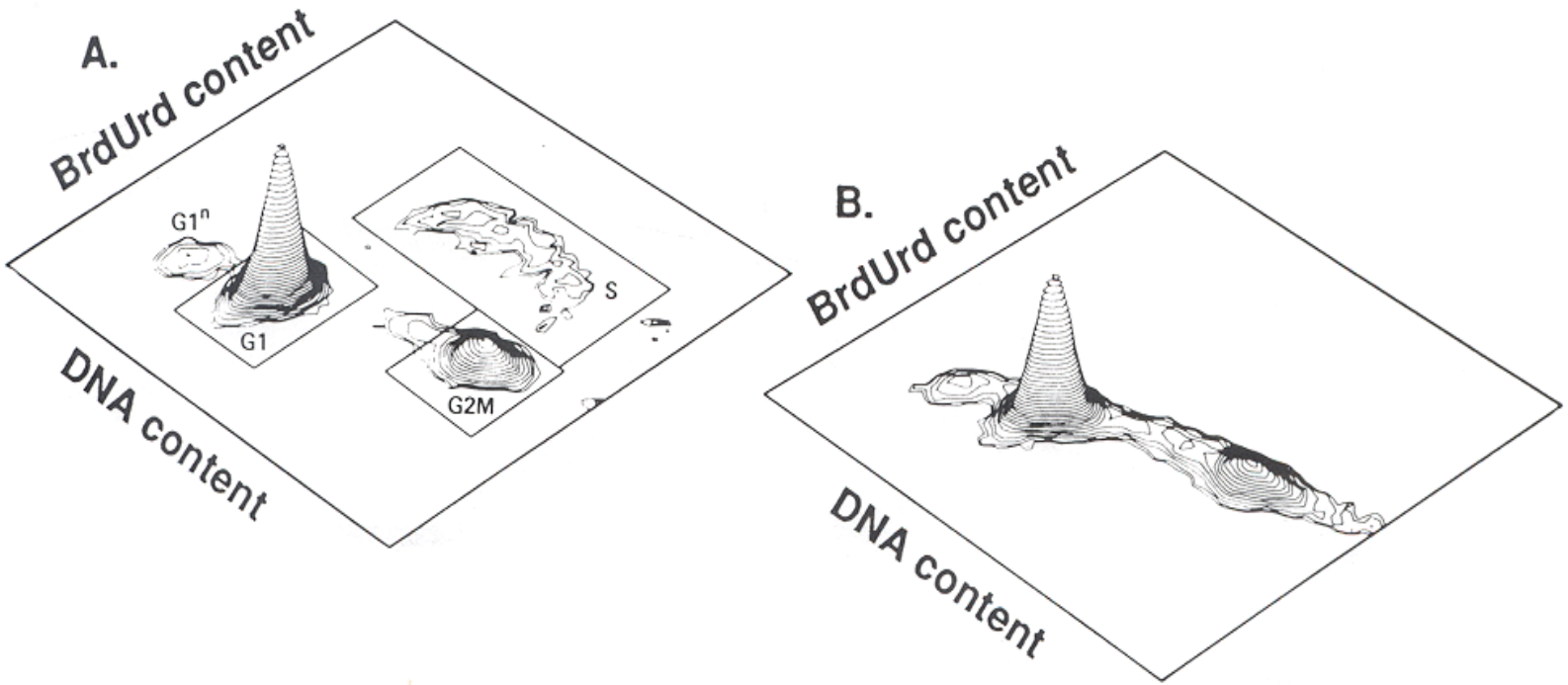
VI. Clinical Application

1. Oncology
2. Hematology (platelet counting, detection of particular leukocyte populations in the blood
3. Immunology
   - Measurement of T cell subpopulations (e.g. HIV)
   - Detection of Ag-specific, activated T cells
   - Intracellular staining for growth factors
   - Evaluation of responses to vaccination: ELIspot *vs*. tetramer-staining

Phenotyping of Human Peripheral Blood Mononuclear Cells

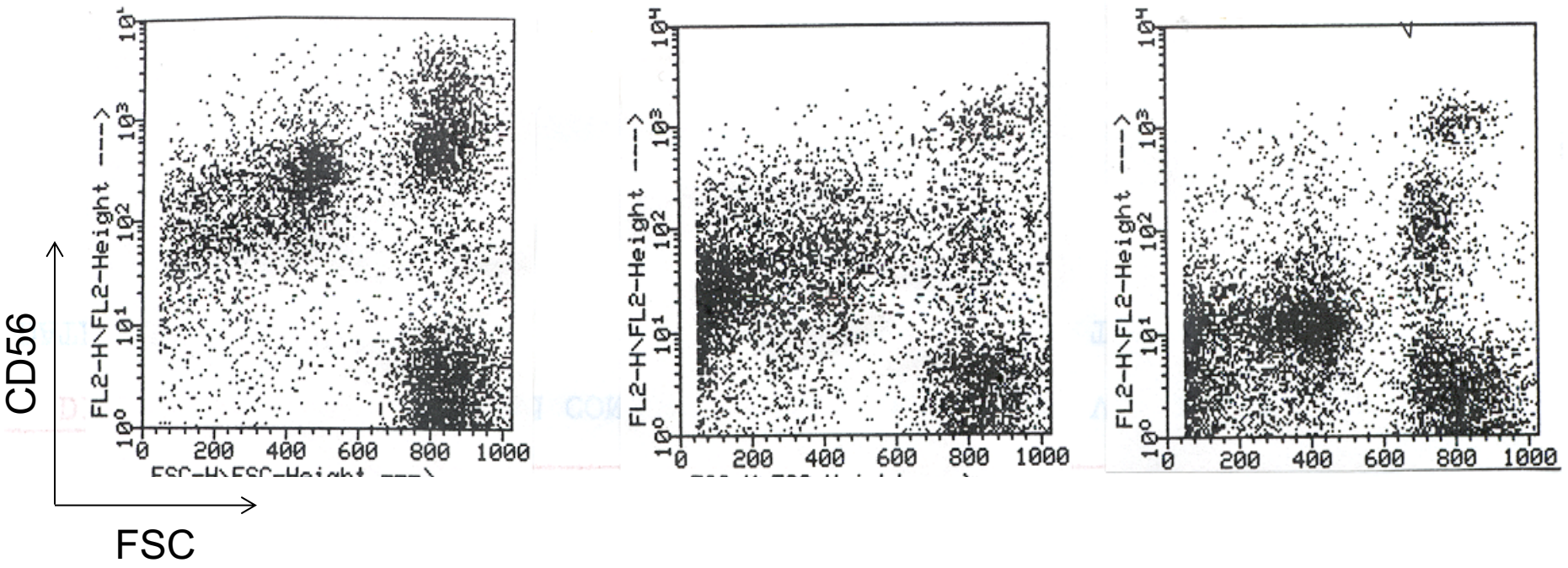


Detection of Proliferating Cells



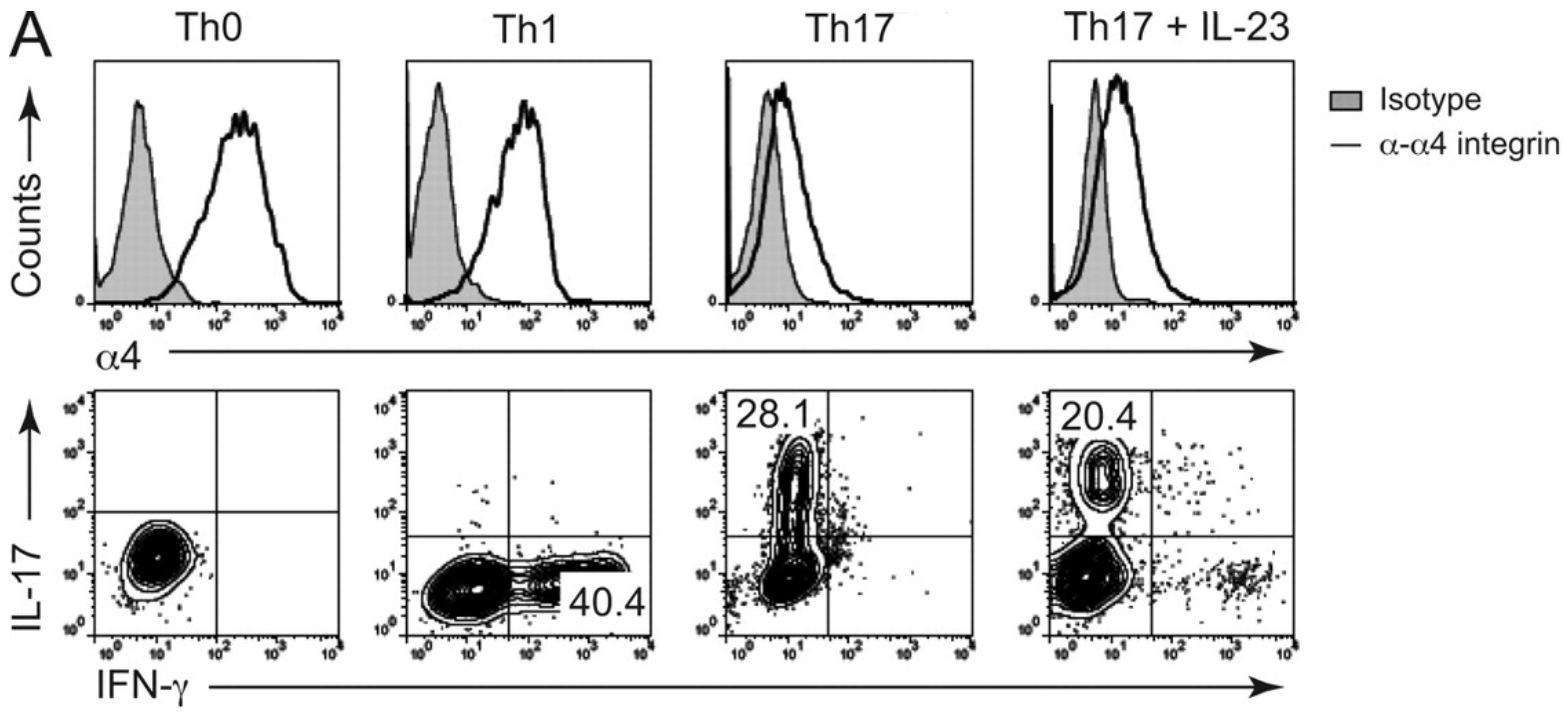
Bivariate distribution (contour plot) of DNA content versus incorporated BrdUrd for human, All cells grown *in vitro*. B: The same type of plot as for panel A, after the cells were treated with ara-c, showing the loss of cells from S-phase.

Detection of Leukocyte Subsets

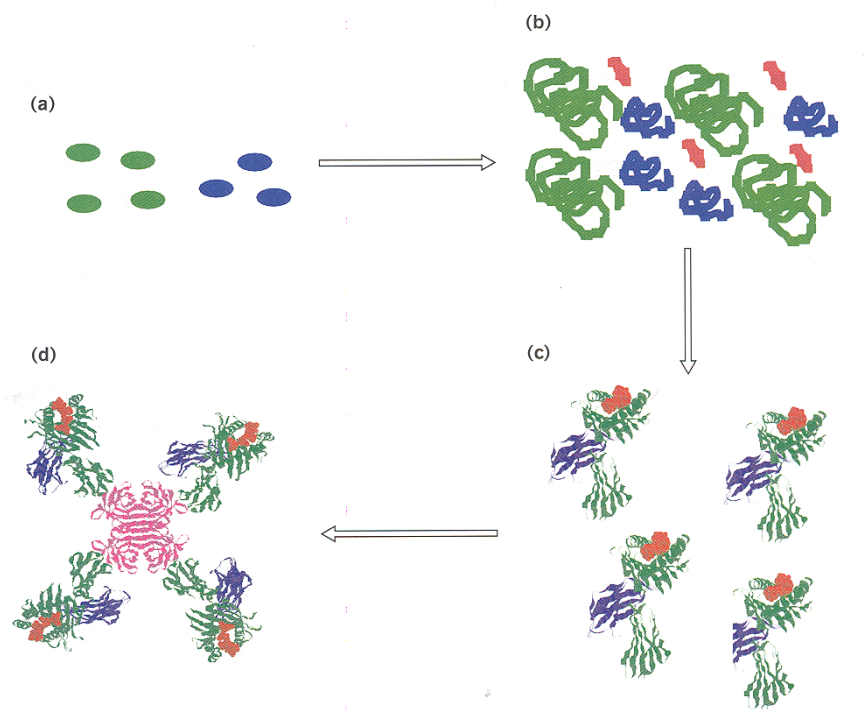


Intracellular Staining for Cytokines

…determines the frequency of cells producing a specific factor (alternate to ELISpots)



Tetramer-staining



(a) Heavy chain is modified by substitution of transmembrane and cytosolic regions with biotinylated target sequence for enzyme BirA

(b) Purification of recombinant proteins form inclusion bodies of E.coli: refolding step *in vitro* and addition of epitope peptide ==> isolation of refolded complexes by FPLC

(c) Loaded complexes are biotinylated at C-terminal end of heavy chain by BirA. Removal of free biotin by FPLC and ion exchange chromatography.

(d) Fluorescent Streptavidin A is added to induce tetramer formation

Other approach: biotinylate all lysine residues of β2m.

Protocol for Staining of Ag-specific T Cells with Tetramers

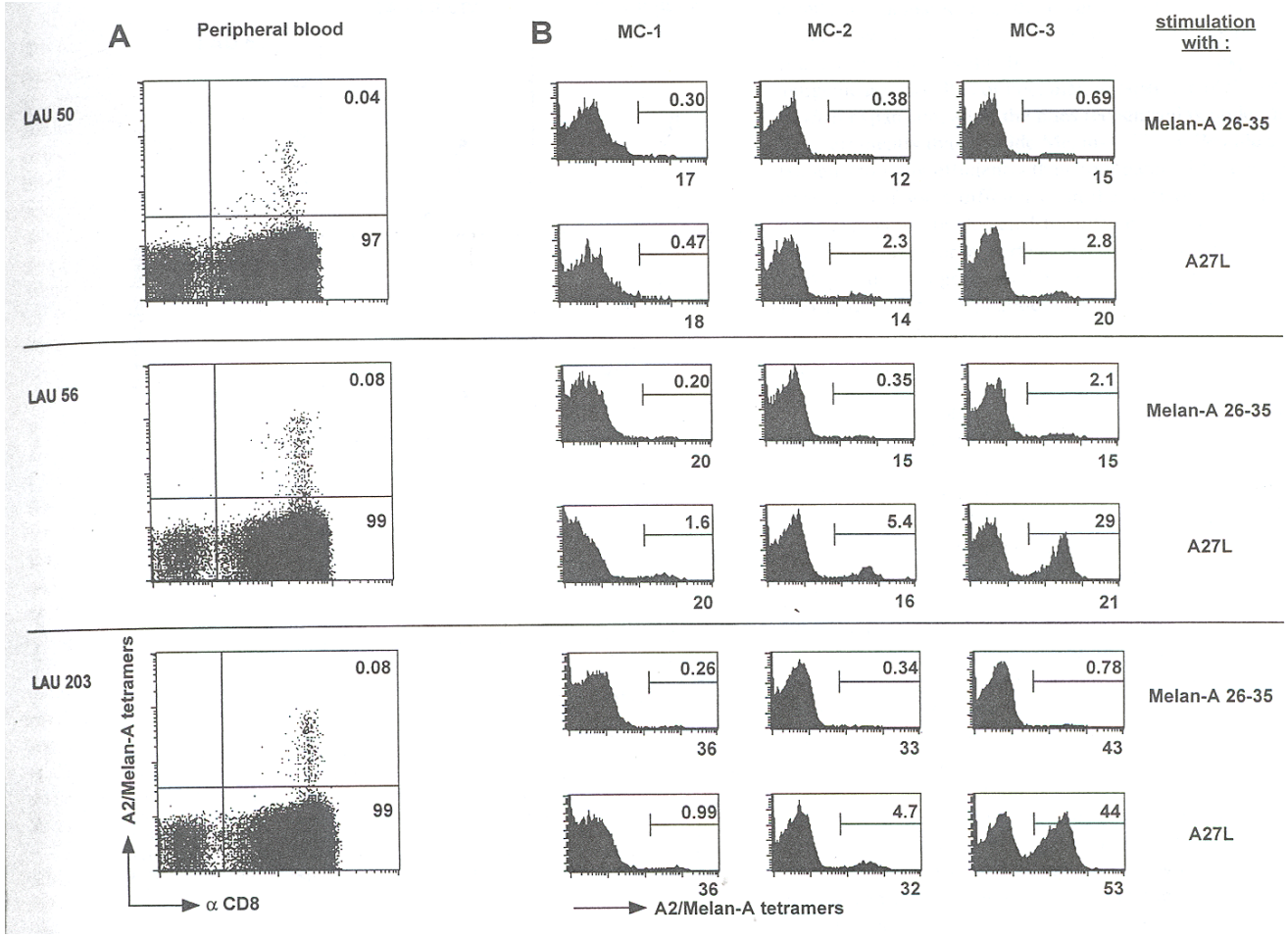
Protocol for Staining of Ag-specific T Cells with Tetramers

- Stain cells in PBS+5%serum with 1mg/ml tetramer for 20min at 4ºC.
- Add anti-CD8 or other mAb (10mg/ml) for 30min at 4ºC.
- Wash cells and analyze in FACS

Gating of Populations Can Increase the Sensitivity of the Analysis

Results based on all PBMC

Results gated on CD8+ T cells



Application of Flow Cytometry in Various Areas of Biochemistry

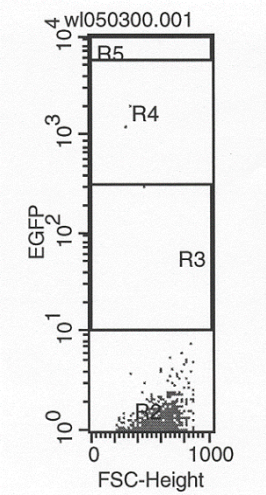
VII. Protein Chemistry

1. Peptide binding to MHC class I or class II (T2 assay, fluorescent peptides)

VIII. Flow Cytometry as an Alternative to Western Blot

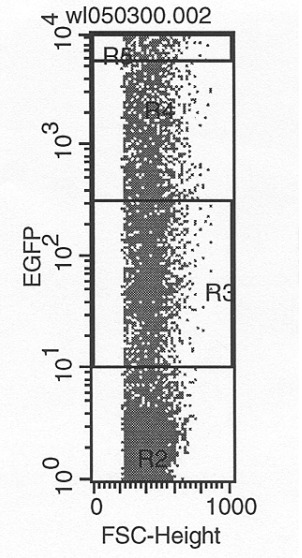
IX. Monitoring the Efficiency of Transfection

Transient Transfection of Cells with a Plasmid



Control (non transfected)

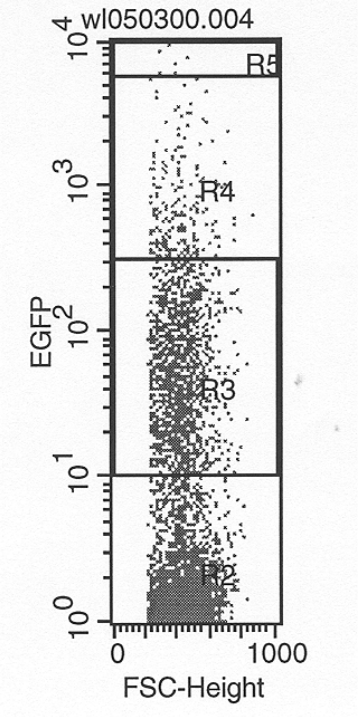
Region	Events	% Gated	% Total	X Mean	X Geo Mean
R1	7971	80.78	72.87	476.66	471.07
R2	9864	99.97	90.18	453.03	443.20
R3	1	0.01	0.01	459.00	459.00
R4	2	0.02	0.02	320.50	320.07
R5	0	0.00	0.00	***	***



Good transfection

Region	Events	% Gated	% Total	X Mean	X Geo Mean
R1	5864	61.64	52.10	464.58	459.60
R2	4893	51.43	43.47	399.93	388.17
R3	2586	27.18	22.98	422.20	408.21
R4	1809	19.01	16.07	431.06	414.40
R5	248	2.61	2.20	441.19	423.34

Transient Transfection of Cells with a Plasmid, Cont'd

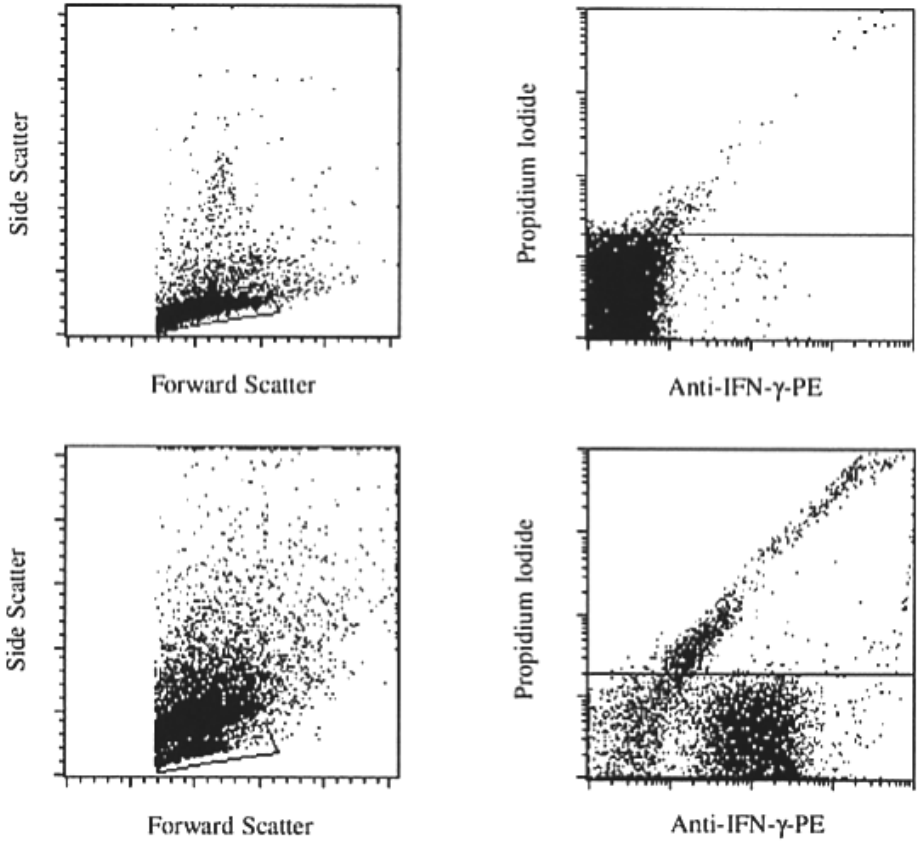


Bad transfection

Region	Events	% Gated	% Total	X Mean	X Geo Mean
R1	5558	58.73	51.65	459.29	454.65
R2	7838	82.83	72.84	395.70	383.46
R3	1401	14.81	13.02	419.14	404.49
R4	230	2.43	2.14	415.95	397.87
R5	7	0.07	0.07	384.71	377.23

Live/Dead Distinction Using FACS Analysis

Eukaryotic cells: Live/dead distinction based on
FSC/SSC characteristics or DNA stains such as PI, 7-AAD etc.



Live/Dead Distinction Using FACS Analysis, Cont'd

Prokaryotic cells: Live/dead distinction based on SSC vs. DIBAC staining